SOP- Modified f/2 Media

1. Introduction and Scope

1.1 This document describes modified f/2 nutrient production methods for indoor seed and outdoor pond production of marine algae species to be used for ATP^3 Unified Field Studies (UFS) and Advanced Field Studies (AFS). Individual stock solutions are to be replenished as needed.

2. Reagents, Materials, and Apparatus Needed

2.1 **Chemicals and Reagents for modified f/2 Media:**

- 2.1.1 Sodium nitrate ($NaNO_3$) or Ammonium Chloride (NH_4Cl)
- 2.1.2 Sodium phosphate monobasic (NaH_2PO_4)
- 2.1.3 Sodium carbonate (Na_2CO_3)
- 2.1.4 Sodium hydroxide (NaOH, can source 1 M solution if preferred)
- 2.1.5 Disodium ethylenediaminetetraacetic acid ($Na_2EDTA*2\,H_2O$)
- 2.1.6 Iron (III) chloride hexahydrate ($FeCl_3*6H_2O$)
- 2.1.7 Copper (II) sulfate pentahydrate ($CuSO_4*5H_2O$)
- 2.1.8 Sodium molybdate dihydrate ($Na_2MoO_4*2H_2O$)
- 2.1.9 Zinc (II) sulfate heptahydrate ($ZnSO_4*7H_2O$)
- 2.1.10 Cobalt chloride hexahydrate ($CoCl_2*6H_2O$)
- 2.1.11 Manganese chloride tetrahydrate ($MnCl_2*4H_2O$)

2.2 **Materials for modified f/2 Media:**

- 2.2.1 (5) 1 L glass bottles
- 2.2.2 (2) 2 L bottles
- 2.2.3 (3) 20 L Nalgene carboys (LDPE) capable of being autoclaved
- 2.2.4 Magnetic stir bars

3. ES&H Considerations and Hazards

3.1 Standard laboratory personal protective equipment shall be worn; lab coat, gloves (nitrile or latex) and eye protection while working with chemicals. Special care should be given when handling sodium nitrate because it is a strong oxidizer. Contact with other materials may cause fire. Harmful if swallowed or inhaled. May cause irritation to skin, eyes and respiratory tract. Special care should be given when handling sodium hydroxide as it is a strong corrosive.

4. Procedure:

4.1 <u>**Generalized Procedure to Prepare Stock Solutions**</u>

- 4.1.1 Prepare all stock solutions in glass bottles.
- 4.1.2 Each stock solution should be prepared and stored separately.
- 4.1.3 Fill one 1L glass bottle approximately halfway with Milli-Q (MQ) or DI water and add a magnetic stir bar.

4.1.4 Place the bottle on a magnetic stir plate and begin stirring the water. Adding chemicals to the bottle while stirring ensures rapid dissolution.
4.1.5 Weigh appropriate amount of chemical(s) and carefully add to the bottle as it is stirring. Use a funnel to aid in transfer if necessary. If a funnel is used, rinse completely with MQ water from a squirt bottle.
4.1.6 Once the chemicals are fully dissolved, remove the bottle from the stir plate and remove the magnetic stir bar.
4.1.7 Volume up to the 1L mark with MQ water.
4.1.8 Autoclave all stock solutions and label each with chemical, name, CAS#, NFPA information and date. Store at room temperature.

4.2 <u>**Preparation of Stock Solutions**</u>

4.2.1 Note that for all of the following it is best to start with a lower volume of MQ water than the final volume calls for and then finish by adding more to reach the final volume after adding chemicals. For all stock solutions, a stir bar can be added to the bottle which is then placed on a stir plate until all solids have dissolved.

4.2.2 **Indoor combined nitrogen and phosphorus stock solution (N:P = 16:1)**

4.2.2.1 Stock solutions can be produced with either: sodium nitrate or ammonium chloride (see Table 1). Produce a separate stock solution for each nitrogen source.
4.2.2.2 Dissolve nitrogen source (see Table 1 for amounts) and 7.5 grams NaH_2PO_4 in MQ water to a total volume of 1 liter.
4.2.2.3 Autoclave and store at room temperature.
4.2.2.4 Use 8.87 ml stock/L culture water.

Table 1: 1 L Stock Solution Indoor Recipes for Various N Sources

N Source	Amount, g	Amount NaH_2PO_4, g
Sodium Nitrate Sln.	85	7.5
Ammonium Chloride Sln.	53.5	7.5

4.2.3 **Outdoor combined N and P stock solution (N:P 16:1)**

4.2.3.1 Stock solutions can be produced with either: sodium nitrate or ammonium chloride (see Table 2). Produce a separate stock solution for each nitrogen source.
4.2.3.2 Dissolve nitrogen source (see Table 2 for amounts) and 7.5 grams NaH_2PO_4 in MQ water to a total volume of 15 liter. The carboy does not need to be sterilized, but should be stored in an air conditioned, low light conditions.

4.2.3.3 Use 1 ml stock/L culture water for initial inoculation. Reference pond protocols for feed levels post harvests.

Table 2: 15 L Stock Solution Outdoor Recipes for Various N Sources

N Source	Amount, kg	Amount NaH_2PO_4, kg
Sodium Nitrate Sln.	6.375	0.5625
Ammonium Chloride Sln.	4.012	0.5625

4.2.4 **Primary trace metal stock solutions**

4.2.4.1 Each of the five primary trace metal stock solutions listed below should be prepared and stored in separate glass bottles.

4.2.4.2 Dissolve 9.8 grams $CuSO_4*5H_2O$ in MQ water to a total volume of 1 liter. Autoclave and store at room temperature.

4.2.4.3 Dissolve 6.3 grams $Na_2MoO_4*2H_2O$ in MQ water to a total volume of 1 liter. Autoclave and store at room temperature.

4.2.4.4 Dissolve 22.0 grams $ZnSO_4*7H_2O$ in MQ water to a total volume of 1 liter. Autoclave and store at room temperature.

4.2.4.5 Dissolve 10.0 grams $CoCl_2*6H_2O$ in MQ water to a total volume of 1 liter. Autoclave and store at room temperature.

4.2.4.6 Dissolve 180.0 grams $MnCl_2*4H_2O$ in MQ water to a total volume of 1 liter. Autoclave and store at room temperature.

4.2.5 **Trace metal stock solution**

4.2.5.1 Begin by dissolving 4.36 grams $Na_2EDTA*2H_2O$ in MQ water.

4.2.5.2 Then, add 3.15 grams $FeCl_3*6H_2O$ and 1.0 mL from each of the five primary trace metal stock solutions [$CuSO_4*5H_2O$ (4.2.3.2), $Na_2MoO_4*2H_2O$ (4.2.3.3), $ZnSO_4*7H_2O$ (4.2.3.4), $CoCl_2*6H_2O$ (4.2.3.5), $MnCl_2*4H_2O$(4.2.3.6)].

4.2.5.3 Continue to stir until fully dissolved.

4.2.5.4 Bring to a final volume of 1 liter with MQ water.

4.2.5.5 Autoclave and store at room temperature.

4.2.5.6 Scale this recipe to 20 L to produce a carboy for outdoor production use. The carboy does not need to be sterilized, but should be stored in air conditioned, low light conditions.

4.2.5.7 Use at 1 ml stock/L culture water.

4.2.6 **Indoor sodium carbonate stock solution (ammonium chloride nitrogen source only)**

4.2.6.1 Dissolve 80 g of sodium carbonate in 1 L DI or milli-Q water. Autoclave and store in an air conditioned space.

4.2.6.2 Use at 12.5 ml/L culture water. Use as needed to maintain pH above 7.0 in culture systems.

4.2.7 **Outdoor sodium hydroxide solution (ammonium chloride nitrogen source only)**

4.2.7.1 Sodium hydroxide is caustic. Wear appropriate PPE.

4.2.7.2 Dissolve 750 g of NaOH in 20 liters of DI water (this is a 1 M solution and can be purchased as a solution as an alternative to producing the solution if preferred). The carboy does not need to be sterilized, but should be stored in air conditioned, low light conditions.

4.2.7.3 Use at 0.25 ml/L culture water at inoculation. Use as needed to maintain pH above 7.0 in culture systems.

END

SOP-Pond General Operations

1. Introduction and Scope

1.1 This document describes the general operations procedure for outdoor ponds at the AzCATI testbed site.

2. Reagents, Materials, and Apparatus Needed

2.1 Outdoor ponds

2.2 YSI 5200 monitoring and control system with probes for pH, temperature, oxidative reduction potential (ORP), dissolved oxygen (DO), conductivity/salinity, and a PAR sensor connected to one YSI unit

3. ES&H Considerations and Hazards

3.1 Standard laboratory personal protective equipment shall be worn including lab coat, nitrile or latex gloves and eye protection, especially while working with chemicals.

3.2 Care should be taken when working around ponds and any electrical equipment, connections. Use caution working around the ponds, including keeping all fingers and equipment away from the paddlewheels.

4. Procedure:

4.1 Prepare inoculum by growing seed algae in columns and then transferring to 2x2 panels (See: **"SOP- Indoor Seed Production in Columns & Panels,"**)

4.2 No more than 24 hours before inoculation, prepare ponds for inoculation (See: **"SOP- Pond Startup and Teardown"**)

4.3 Inoculate ponds using experimental protocol.

4.4 During pond operations, sample ponds with frequency outlined in experimental plan. Use the sampling and handling procedures outlined in **"SOP- Pond Wet Sample Acquisition, Handling, Storage & Analytical Sample Processing."**

4.5 Perform sample tests or preparation as outlined in experimental plan. All or some of the following tests will be performed at different time points throughout the duration of the experiment:

4.5.1 Ash Free Dry Weight (See: **"SOP- Gravimetric Method for Determination of Dry Weight (DW) and Ash Free Dry Weight (AFDW)"**)

4.5.2 Optical Density (See: **"SOP- Optical Density**)

4.5.3 Nitrate Assay (See: **"SOP- Preparation of Samples for Nitrate Analysis (Lachat QuikChem 8500)"**)

4.5.4 Phosphate Assay (See: **"SOP- Preparation of Samples for Phosphate Analysis (Lachat QuikChem 8500)"**)

4.5.5 Ammonia Assay (See: **"SOP- Preparation of Samples for Ammonia Analysis (Lachat QuikChem 8500)"**)

4.5.6 Microscopy (See: **"SOP- Quick Contamination Check"**)

4.5.7 Molecular Fingerprint (See: **"SOP- Genetic Sampling Based on Ash Free Dry Weight"**)

4.5.8 Analytical Sample Processing (See: **"SOP- Pond Wet Sample Acquisition, Handling, Storage & Analytical Sample Processing,"**) 4.5.9 Wait until ponds reach desired density on grow-out according to experiment protocol (no less than 0.3g/L) and, if applicable, execute pond harvest operations using experiment protocol.

4.5.10 Determine whether or when ponds will be run until deplete and consider nutrient uptake in order to determine how much N:P stock during experiment, either at harvest or otherwise to keep replete. Nutrient uptake may vary with geographic location and season.

4.5.11 Determine pond termination date based on experiment.

4.5.12 After termination of experiment, algae culture will be harvested or killed and dumped, and ponds will be cleaned. (See "**SOP- Pond Startup and Teardown,"**)

END

SOP-Pond Startup and Teardown

1. Introduction and Scope

1.1 This document describes the process of cleaning and setting up the outdoor ponds at the AzCATI testbed site including the teardown of the experiment, with proper storage of sensitive equipment and contained disposal of residual algae.

2. Reagents, Materials, and Apparatus Needed

2.1 **Materials for Raised Pond Preparation for Inoculation:**

2.1.1 Outdoor ponds

2.1.2 YSI 5200 monitoring and control system with probes for pH, temperature, oxidative reduction potential (ORP), dissolved oxygen (DO), conductivity/salinity, and a PAR sensor connected to one YSI unit

2.1.3 Household bleach or equivalent sodium hypochlorite solution (6%, 10% or 12%), roughly one gallon per pond

2.1.4 Water source

2.1.5 Oceanic Sea Salt, or equivalent (if applicable), roughly 35kg per pond

2.2 **Materials for Pond Teardown:**

2.2.1

2.2.2 Light-duty pressure washer (below 2000PSI)

2.2.3 Household bleach or equivalent sodium hypochlorite solution (6%, 10% or 12%)

2.2.4 Pool-grade Muriatic Acid, roughly 1L per pond

2.2.5 Water source

2.2.6 Scour pad

2.2.7 Rubber gloves

2.2.8 Sodium thiosulfate

2.2.9 Chlorine test kit or test strips from local pool supply or aquarium store

2.2.10 Label printer and labels (for biochemical composition, harvest, and molecular sample tracking)

2.2.11 Graduated cylinders

2.2.12 Scrubbing brush for use with muriatic acid only

3. ES&H Considerations and Hazards

3.1 Standard laboratory personal protective equipment shall be worn including lab coat, nitrile or latex gloves and eye protection, especially while working with chemicals. Special care should be given when handling sodium nitrate because it is a strong oxidizer. Special care should be given when handling sodium hypochlorite because it is corrosive and a strong irritant.

3.2 Extreme care should be exercised when using muriatic acid, an acute health hazard. You should be in a well-ventilated area while handling muriatic acid, and additional PPE must be worn such as face mask, protective rubber gloves, and safety glasses with a side shield or goggles. Contact with other materials may cause fire. Harmful or fatal if swallowed. Harmful if inhaled and may cause burns or irritation to skin, eyes and respiratory tract.

3.3 Care should be taken when working around ponds and any electrical equipment, connections. Use caution working around the ponds, including keeping all fingers and equipment away from the paddlewheels.

4. Procedure:

4.1 Raised Pond Preparation for Inoculation

4.1.1 Rinse all ponds and paddlewheels thoroughly of dust and other residue that may have collected in empty pond. If the pond is not yet empty and you are disposing of experimental cultures, refer to pond teardown procedure in procedures under step 4.2 of this procedure.

4.1.2 Making sure sparger is suspended in water and not already in the pond, perform a surface sterilization of rinsed pond with 16:1 solution of water: household bleach (Chlorox brand or equivalent without scent). Rinse thoroughly.

4.1.3 Flush all water lines and filters prior to collecting water for cultivation purposes for at least 5 minutes.

4.1.4 Type of water used is strain dependent. Fill to one centimeter below desired experimental depth (read: to 24 cm for a 25 centimeter final depth with a nominal volume of 1000L). If creating artificial seawater in your experiment, add salt, let salt dissolve, and then verify salinity with YSI salinity probe. For standard seawater, use 35g/L sea salt (35ppt). If using natural seawater, verify salinity with YSI salinity probe and adjust with artificial sea salt or water to achieve desired salinity. **NOTE: Pond drain plugs are prone to degradation, so you must make sure that the drain is not leaking before continuing with the procedure. If drain plug is leaking, it is recommended to either replace the plug or incase in a nitrile glove.**

4.1.5 Sterilize culture water in the pond (system and water sterilization) with sodium hypochlorite (bleach). Use 1 ml 12% bleach solution/L water, 1.6 ml 10% bleach solution/L water, 2 ml 6% bleach solution/L water to achieve a 100 ppm (100 mg/L) chlorine concentration for at least 12 hours, but no more than 24 hours. Follow "**UFS Spring 2015 Experiment Protocol**" 5.4 for pond inoculation procedure. **NOTE: If more than 24 hours passes between pond preparation and pond inoculation, you must repeat 4.1.1-4.1.5 to prepare ponds for inoculation.**

4.1.6 Immediately before inoculation, CO_2 sparger should be deployed. Repeat sanitization step outlined in **4.2.2**, rinse thoroughly and deploy.

4.1.7 Check ponds for chlorine using chlorine test strips. If chlorine residual remains, dechlorinate with 0.01 ml/L culture water of sodium thiosulfate stock solution (dissolve 500 g sodium thiosulfate in 1 L DI or milli-Q water). Verify dechlorination is complete with a chlorine test kit after 10 minutes. If dechlorination is not complete add 0.01 ml/L sodium thiosulfate stock solution, wait 10 minutes and test again with the chlorine test kit. Repeat this process until dechlorination is complete. You should not use more than 0.2ml/L sodium thiosulfate stock to dechlorinate.

4.1.8 Clean YSI probes need to be attached and calibrated using **"SOP- YSI Control System Protocol."** If any delays are observed, YSI must be stored vertically in pH4 buffer before deployment, but should not be withheld from use outside for more than 24 hours.

4.2 Pond Teardown

4.2.1 Remove YSI probe, clean and store as described in **"SOP-YSI Control System Protocol."**

4.2.2 Remove CO_2 sparger and tube and place in a bucket separate from the pond. You will need to clean the sparger separately from the pond because it must be cleaned with a higher concentration of sodium hypochlorite (1000ppm for 2 min) for sanitation. Scrub it clean of algae and rinse immediately. **NOTE: The CO_2 sparger should not be left in bleach in the pond or it will degrade and sparging bubbles will be much larger than intended for the experiment, causing inefficient CO_2 delivery and uptake.**

4.2.3 Fill pond to 1cm above previous fill depth for ease of cleaning.

4.2.4 Use 1 ml 12% bleach solution/L water, 1.6 ml 10% bleach solution/L water, 2 ml 6% bleach solution/L water to achieve a 100 ppm (100 mg/L) chlorine concentration for 12 hours with paddlewheel on.

4.2.5 After bleaching the algae, it may be disposed of down the sewer by pulling the drain plug. Drain plug should be rinsed clean of bleach and stored indoors to prevent sun damage.

4.2.6 Use a combination of scour pads and the low intensity power washer to scrub away any remaining algae residue on pond and paddlewheel and rinse away bleach. **NOTE: You must wear goggles and other protective gear while using the pressure-washer on bleached materials.**

4.2.7 If walls of pond is stained at all from experimental culture, you must clean with concentrated muriatic acid to ensure uniform reflective surfaces in all of your experimental replicates. It is recommended to use a separate scrubbing brush for use with muriatic acid only. *****ATTENTION*** Throughout this procedure take utmost care in personal protective equipment, including goggles, face mask or shield, and rubber gloves as muriatic acid is an acute health hazard. It should not be inhaled, and should not come into contact with skin.**

4.2.8 Rinse pond thoroughly of muriatic acid and leave empty.

4.2.9 Repeat 4.2.1-4.2.8 for each pond to be torn down.

END

SOP-Pond Wet Sample Acquisition, Handling, Storage & Analytical Sample Processing

1. Introduction and Scope

1.1 This document describes the grab sample procedure from outdoor ponds and wet sample handling at the AzCATI testbed site. Wet sample acquisition, wet sample storage, and processing procedure for analytical grab samples are contained herein.

2. Reagents, Materials, and Apparatus Needed

2.1 **Materials: Sample acquisition from ponds and pond monitoring procedure**

- 2.1.1 Outdoor ponds
- 2.1.2 YSI 5200 monitoring and control system with probes for pH, temperature, oxidative reduction potential (ORP), dissolved oxygen (DO), conductivity/salinity, and a PAR sensor connected to one YSI unit
- 2.1.3 Handheld pH meter
- 2.1.4 Handheld thermometer
- 2.1.5 Handheld salinity meter
- 2.1.6 70% Ethanol solution
- 2.1.7 Laboratory tape
- 2.1.8 Sample bottles and tubes (varying sizes per sample draw volume). Recommended sample volumes: 1L, 2L, 250mL, 50mL

2.2 **Materials: Wet Sample Storage**

- 2.2.1 Refrigerator set to 40°F
- 2.2.2 An opaque drawer or container inside refrigerator

2.3 **Materials: Analytical Grab Sample Processing**

- 2.3.1 Grinding vial set (5-Well 15mL Polycarbonate Vials, unlined cap, with 11.1cm (7/16”) Grinding balls)
- 2.3.2 High Throughput Homogenizer
- 2.3.3 Laboratory scraper or spatula
- 2.3.4 Freeze-drier
- 2.3.5 Laboratory tape
- 2.3.6 -80°F Freezer
- 2.3.7 Thermal gloves rated for -80°F
- 2.3.8 Beckman-Coulter Avanti J Centrifuge series and JLA 8.1000 fixed-angle rotor 1L bottle assembly (or similar) **NOTE: Centrifuges holding smaller volumes (50mL, 250mL) may be used but many more supernatant dumps and refills will be necessary for processing large volumes and it is not recommended.**

3. ES&H Considerations and Hazards

3.1 Standard laboratory personal protective equipment shall be worn including lab coat, nitrile or latex gloves and eye protection, especially while working with chemicals.

3.2 Care should be taken when working around ponds and any electrical equipment, connections. Use caution working around the ponds, including keeping all fingers and equipment away from the paddlewheels.

4. Procedure:

4.1 Sample acquisition from outdoor ponds and pond monitoring procedure

4.1.1 Samples should be acquired Mon-Friday at Sunrise ± 30 min

4.1.2 Turn off paddlewheel on pond containing algae culture

4.1.3 Record depth with affixed ruler and record pH and temp with handheld sensors. If saltwater, record salinity with handheld salinity meter. **NOTE: Make sure handheld sensors are calibrated before recording. Check to make sure YSI control panel is reading like numbers to calibrated handheld sensors. If values do not match, refer to "YSI Control System Protocol" and recalibrate or replace YSI probes as needed. If pH on handheld and YSI agree but are outside of acceptable range for experiment, consult "Pond General Operations Protocol" to troubleshoot CO_2 delivery.**

4.1.4 Replace evaporative loss of water if under desired experimental depth. Flush water lines for five to ten minutes before using lines to add to pond.

4.1.5 Turn paddlewheel on and allow 10 minutes mixing.

4.1.6 Using a clean glove and clean sample containers, dip containers after the paddlewheel and fill samples from roughly the middle of the water column. **NOTE: Amount of sample drawn is directly related to density of the algae culture and tests performed. See Table 1 below to calculate sample volume.**

4.1.7 Rinse handheld sensors immediately before returning to pond or back into their respective caps.

4.1.8 Repeat Steps 4.4.2 through 4.1.7 until all ponds are sampled.

*****ATTENTION*** Throughout this procedure take utmost care to keep individual ponds clean and separate by rinsing handheld devices thoroughly, changing gloves often, and spraying occasionally with 70% ethanol. It is important that sample jars remain clean and separate until all samples have been drawn to avoid cross-contamination.**

Table 1: Minimum wet sample volume needed for experiments based on algae density

	0.05g/L	0.10g/L	0.15g/L	0.20g/L	0.25g/L	0.3g/L
Analytical Grab	6.5L	3.5L	2.5L	2L	1.7L	1.5L
Ash Free Dry Weight	300mL	150mL	100mL	75mL	60mL	50mL
Optical Density	3mL	3mL	3mL	3mL	3mL	3mL
NO_3	6mL	6mL	6mL	6mL	6mL	6mL
PO_4	15mL	15mL	15mL	15mL	15mL	15mL
NH_4	15mL	15mL	15mL	15mL	15mL	15mL
Microscopy	1mL	1mL	1mL	1mL	1mL	1mL
Molecular	100mL	100mL	100mL	100mL	100mL	100mL

4.2 Procedure for Wet sample Storage

4.2.1 Wet samples must be stored in the dark in the cold (40°F) when not actively being processed. If in a shared refrigeration space, keep in a dark container to ensure wet samples are kept in the dark at all times when not being processed.

4.2.2 Processing may continue for up to a month for most experiments while samples are stored in the cold in the dark without any noticeable change to experiments **with the exception of microscopy, which must be done before samples are stored and NO_3 PO_4 NH_4 processes of separating supernatant from biologicals, which should be done the day they are drawn (read: or as soon as possible) to avoid nutrient uptake past sample time point.**

4.3 **Procedure for Analytical Grab Sample Processing**

*****ATTENTION*** Thermal gloves must be used in addition to basic laboratory PPE when using the -80 freezer and handling frozen sample vials.**

4.3.1 Place clean 1L centrifuge bottle and cap for Beckman-Coulter Avanti J Centrifuge series and JLA 8.1000 fixed-angle rotor on analytical balance capable of measuring 1.5kg.

4.3.2 Label bottle with sample information and pour algae up to the 1L mark without exceeding 1.5kg. Record the final weight of the cylinder.

4.3.3 Weigh five identical weight 1L assemblies (use DI water if running less than six samples at once)

4.3.4 Using the Beckman-Coulter Avanti J Centrifuge procedures, spin at 6,000 RPM for ten minutes at room temperature

4.3.5 Remove bottle when finished and pour out as much supernatant from the sample as you can without losing any pellet. Weighing into same sample container, repeat steps 4.3.2 through 4.3.5 until sample volume is exhausted.

4.3.6 Label 15mL polycarbonate vials for each sample centrifuged and record in log book

4.3.7 Use a clean scoop or other scraping tool to transfer pellet to 15mL vial. **In the event that the sample only forms a partial pellet or breaks apart, you may choose to re-suspend partial pellet in a smaller volume and centrifuge in a 50mL tube, which will consolidate more easily than the larger, 1L bottle.**

4.3.8 Place 15mL vial in -80 freezer overnight, or until frozen through completely.

4.3.9 After frozen, remove vial from freezer and crack lid to allow air flow.

4.3.10 Quickly place frozen 15mL vial with biomass on freeze-drier in freezer-dry tray or flask and follow procedure for freeze-drier operation to freeze-dry pellet.

4.3.11 Remove 15mL vial when sample is completely freeze-dried (likely over 1-2 days depending on strength of freeze drier and number of samples). You will be able to tell if a sample is done freeze-drying when the flask or tray is room temperature.

4.3.12 Place two 11.1cm grinding balls in each 15mL vial, cap, and set in a 5-well homogenization tray. Make sure all five vials are present in the tray for balance and run on high-throughput homogenizer on medium strength for five minutes.

4.3.13 Transfer homogenized sample to sample bag with a comprehensive label and store in 40°F in the dark. **NOTE: If sample biomass does not appear completely homogenized, run again on the homogenizer. If samples seem sticky or wet, they have not been correctly freeze-dried. Make note of the state of sample and return to step 4.3.8 to correctly freeze-dry sample.**

END

IOP- Determination of ammonia (phenolate), ortho-phosphate, and nitrate/nitrite in water by flow injection analysis colorimetry

1. Introduction and Scope

1.1 The purpose of this method is three-fold: 1) to determine ammonia (phenolate) in algal supernatant. It is based on the reaction of the ammonium (NH_4^+) ion, 2) to determine orthophosphate in algal supernatant, and 3) to determine nitrate/nitrite in algal supernatant.

2. Summary of Method

2.1 The ammonium ion (NH_4^+) reacts with alkaline phenolate, then with sodium hypochlorite to form indophenol blue. Sodium nitroprusside is added to enhance sensitivity. The absorbance of this reaction is measured at 630 nm. The absorbance is directly proportional to the original ammonia concentration in the sample. The applicable range is 0.1 to 30.0 mg N/L as NH_4^+. The statistically determined detection limit as determined in water is 0.01 mg N/L. The method throughput is approximately 90 injections per hour.

2.2 The orthophosphate ion (PO_4^{3-}) reacts with ammonium molybdate and antimony potassium tartrate under acidic conditions to form a complex. This complex is reduced with ascorbic acid to form a blue complex which absorbs light at 880 nm. The absorbance is proportional to the concentration of orthophosphate in the sample. The applicable range is 0.01 to 2.00 mg P/L as PO_4^{3-}. The statistically determined detection limit as determined in water is 0.01 mg P/L. The method throughput is approximately 90 injections per hour.

2.3 The nitrate (NO_3^-) is quantitatively reduced to nitrate (NO_2^-) by passage through a copperized cadmium column. The nitrite (reduced nitrate plus original nitrite) is then determined by diazotizing with sulfanilamide followed by coupling with N-(1-naphthylethylenediamine dihydrochloride [NED]. The resulting water soluble dye has a magenta color which is read at 520 nm. The absorbance is proportional to the concentration of nitrate/nitrite in the sample. Nitrite alone also can be determined by running without the cadmium column. The applicable range is 0.2 to 20.0 mg N/L as NO_3^- or NO_2^-. The statistically determined detection limit as determined in water is 0.01 mg N/L. The method throughput is approximately 55 injections per hour.

3. Reagents, Standards, Materials, and Apparatus Needed

3.1 Chemicals and Reagents:

3.1.1 Preparation of reagents for the determination of ammonia (phenolate).

REAGENT 1: Sodium Phenolate
In a 1000-mL volumetric flask, dissolve 83.0g of crystalline phenol (C_6H_5OH) in approximately 600-mL deionized (DI) water. While stirring, slowly add 32.0g of sodium hydroxide (NaOH). Cool, dilute to the mark, and invert to mix. Do not degas this reagent. Prepare fresh every 3-5 days. Discard when reagent turns dark brown. Wrap in foil or store in a dark bottle and refrigerate to store.

REAGENT 2: Sodium Hypochlorite

In a 500-mL volumetric flask, dilute 218-mL of 6% sodium hyplochlorite (NaOCl) to the mark with DI water. Invert to mix. Prepare fresh every month.

REAGENT 3: EDTA Buffer
In a 1000-mL volumetric flask, dissolve 55.36g of disodium ethylenediamine tetraacetic acid dihydrate (Na2EDTA·2H2O) and 5.5g of sodium hydroxide (NaOH) in approximately 900-mL DI water. While stirring, slowly add 32.0g of sodium hydroxide (NaOH). Dilute to the mark and mix with a magnetic stirrer until dissolved. Prepare fresh every month.

REAGENT 4: Sodium Nitroprusside
In a 1000-mL volumetric flask, dissolve 3.5g of sodium nitroprusside (sodium nitroferricyanide [$Na_2[Fe(CN)_5NO]\cdot 2H_2O$]). Dilute to the mark, and invert to mix. Prepare fresh every 1-2 weeks. Wrap in foil or store in a dark bottle and refrigerate to store.

REAGENT 5 – 10% Hydrochloric Acid Rinse
In a hood, to a 1000-mL container, add 500-mL DI water and 100-mL of concentrated hydrochloric acid (HCl). Dilute to mark (1000-mL). Invert to mix.

3.1.2 Preparation of reagents for the determination of orthophosphates.

REAGENT 1 - Stock Ammonium Molybdate Reagent
In a 1000-mL volumetric flask, add 800-mL deionized water and 40.0 g ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$). Dilute to mark and stir for 4 hours. Store in a plastic container and refrigerate. May be stored up to 2 months when kept refrigerated.

REAGENT 2 - Stock Antimony Potassium Tartrate Solution
In a 1000-mL volumetric flask, add 800-mL DI water and 3.22 g antimony potassium tartrate trihydrate ($C_8H_4O_{12}K_2Sb_2\cdot 3H_2O$). Dilute to mark and invert 3 times to mix. Store in a dark bottle and refrigerate. May be stored up to 2 months when kept refrigerated.

REAGENT 3 - Molybdate Color Reagent
In a 1000-mL volumetric flask, add 500-mL DI water and 35.0-mL concentrated sulfuric acid. CAUTION: Solution will be hot. Swirl to mix. When the solution can be comfortably handled, add 72.0-mL of the Stock Antimony Potassium Tartrate Solution and 213.0-mL of the Stock Ammonium Molybdate Solution. Dilute to mark and invert 3 times to mix. Prepare fresh weekly. Discard if solution turns blue.

REAGENT 4 - Ascorbic Acid Reducing Solution
In a 1000-mL volumetric flask, add 700-mL DI water, 60.0 g ascorbic acid. Dilute to mark and invert to mix. Add 1.0 g dodecyl sulfate ($CH_3(CH_2)_{11}OSO_3Na$). Invert to mix. Discard when solution turns yellow. Prepare fresh weekly. Note: For maximum analysis sensitivity, use ascorbic acid powder within one year of receipt.

REAGENT 5 - Sodium Hydroxide - EDTA Rinse
To a 1000-mL container, add 900-mL DI water, 65.0 g sodium hydroxide (NaOH) and 6.0 g tetrasodium ethylenediamine tetraacetic acid (Na_4EDTA). Dilute to mark (1000-mL). Stir until dissolved.

3.1.3 Preparation of reagents for the determination of nitrate/nitrite.

REAGENT 1 – 15 N Sodium Hydroxide
Add 150g of sodium hydroxide (NaOH) very slowly to 250-mL or g of DI water. CAUTION: The solution will get very hot! Swirl until dissolved. Cool and store in a plastic bottle.

REAGENT 2 - Ammonium Chloride Buffer, pH 8.5
In a hood a 1000-mL volumetric flask, add 500-mL DI water, 105-mL of concentrated hydrochloric acid (HCl), 95-mL of ammonium hydroxide (NH4OH), and 1.0g disodium EDTA. Dissolve and dilute to the mark. Invert to mix. Adjust pH to 8.5 with HCL or 15 N NaOH solution

REAGENT 3 - Sulfanilamide Color Reagent
In a 1000-mL volumetric flask, add approximately 600-mL DI water, 100-mL of 85% phosphoric acid (H3PO4), 40.0 g sulfanilamide, and 1.0g N-(1-naphthyl) ethylenediamine dihydrochloride (NED). Shake to wet, and stir to dissolve for 30 minutes. Dilute to mark and invert to mix. Store in a dark bottle. May be stored up to 1 month.

3.2 **Standards:**

3.2.1 Preparation of Standards for the determination of ammonia (phenolate).

Standard 1: Stock Ammonia Standard 1000mg N/L as NH_3
In a 100-mL volumetric flask, dissolve 0.3819g of ammonium chloride (NH_4Cl) that has been dried for two hours at 110 degrees C, in about 50-mL DI water. Dilute to the mark and invert to mix.

Standard 2: Stock Ammonia Standard 200mg N/L as NH_3
In a 100-mL volumetric flask add 20-mL of Stock Standard 1 (1000mg N/L). Dilute to the mark with DI water and invert to mix.

Working Standards (prepare daily)	A	B	C	D	E	F	G
Concentration mg N/L as NH_3	30.0	15.0	5.0	1.00	0.50	0.10	0.00
Volume (mL) of Stock Standard 2 diluted to 100-mL with DI water	15.0	7.5	2.5	0.5	0.25	0.05	0.0
Volume (mL) of Standard C diluted to 100-mL with DI water	---	---	---	20.0	10.0	2.0	0.0

3.2.2 Preparation of Standards for the determination of orthophosphates.

Standard 1: Stock Phosphate Standard 250.0mg P/L as PO_4^{3-}
In a 100-mL volumetric flask, dissolve 0.1099 g potassium phosphate monobasic (KH_2PO_4) to the mark with deionized water. Invert to mix. This standard stock solution is 250.0 mg P/L as PO_4^{3-}. Store in a plastic container wrapped in foil. Keep refrigerated. Can be stored up to 1 month when kept refrigerated.

Standard 2: Stock Phosphate Standard 5.00mg P/L as PO_4^{3-}
In a 100-mL volumetric flask, dilute 2.0-mL of PRIMARY STOCK STANDARD to the mark with deionized water. Invert to mix. This standard stock solution is 5.00 mg P/L as PO43-. Prepare fresh daily.

Working Standards (prepare daily)	A	B	C	D	E	F
Concentration mg P/L as PO_4^{3-}	2.00	0.50	0.20	0.05	0.01	0.00
Volume (mL) of Stock Standard 2 diluted to 100-mL with DI water	40.0	10.0	4.0	1.0	0.2	0.0

3.2.3 Preparation of Standards for the determination of nitrate/nitrite

Standard 1: Stock Nitrate Standard 200mg N/L as NO_3^-
In a 500-mL volumetric flask, dissolve 0.722g of potassium nitrate (KNO_3) in about 300-mL DI water. Dilute to the mark and invert to mix. This solution is stable for 6 months. Wrap in foil and refrigerate to store.

Working Standards (prepare daily)	A	B	C	D	E	F	G
Concentration mg N/L as NO_3	20.0	8.0	4.0	1.00	0.40	0.20	0.00
Volume (mL) of Stock Standard 1 diluted to 100-mL with DI water	10.0	4.0	2.0	0.5	.02	.01	0.0
Volume (mL) of Standard A diluted to 100-mL with DI water	---	---	---	5.0	2.0	1.0	0.0

Standard 2: Stock Nitrite Standard 200mg N/L as NO_2^-
In a 500-mL volumetric flask, dissolve 0.493g of sodium nitrite ($NaNO_2$) or .607g of potassium nitrite (KNO_2) in about 400-mL DI water. Dilute to the mark and invert to mix. This solution is stable for 3-5 days. Wrap in foil and refrigerate to store.

Working Standards (prepare daily)	A	B	C	D	E	F	G
Concentration mg N/L as NO_2	20.0	8.0	4.0	1.00	0.40	0.20	0.00
Volume (mL) of Stock Standard 2 diluted to 100-mL with DI water	10.0	4.0	2.0	0.5	.02	.01	0.0
Volume (mL) of Standard A diluted to 100-mL with DI water	---	---	---	5.0	2.0	1.0	0.0

3.3 **Materials:**

3.3.1 15-mL conical tube (plastic)
3.3.2 Volumetric flasks (100-mL, 500-mL, and 1000-mL)
3.3.3 Graduated cylinders (100-mL, 250-mL, 500-mL, 1000-mL)
3.3.4 Pipettes (100µL-1000µL, 5-10 mL)
3.3.5 Pipette tips (100µL-1000µL, 5-10 mL)
3.3.6 3L flasks

3.4 **Apparatus:**

3.4.1 Balance -- analytical, capable of accurately weighing to the nearest 0.0001g.
3.4.2 Glassware – Class A volumetric flasks and pipettes or plastic containers as required. Samples may be stored in plastic or glass.
3.4.3 Flow injection analysis equipment designed to deliver and react sample and reagents in the required order and ratios.
3.4.3.1 Sampler
3.4.3.2 Multichannel proportioning pump
3.4.3.3 Reaction unit or manifold
3.4.3.4 Colorimetric detector
3.4.3.5 Data system
3.4.4 Acid-washed glassware: All glassware used in the determination of phosphate should be washed with hot 1:1 HCl and rinsed with distilled water. Preferable this glassware should be used only for the determination of phosphates and after use, it should be rinsed with

distilled water and kept covered until needed again. If this is done, the treatment with 1:1 HCl is only required occasionally. Commercial detergent should never be used.

3.4.5 Flow cell

3.4.6 520nm, 630nm, 880nm interference filter

3.4.7 Special apparatus

3.4.7.1 Heating Unit, Lachat part No. A85X00 (X=1 for 110V, X=2 for 220V) for phosphate and ammonia determination.

3.4.8 PVC pump tubes must be used for ammonia determination method.

4. ES&H Considerations and Hazards

4.1 Standard laboratory personal protective equipment shall be worn; lab coat, gloves (nitrile or latex) and eye protection while working with chemicals.

4.2 The chemical used to prepare the sodium hypochlorite reagent is sodium hypochlorite (NaClO). Sodium hypochlorite is an irritant to both the respiratory track and dermis. Please read the chemical's MSDS before using.

4.3 The chemical used to prepare the sodium nitroprusside reagent is sodium nitroprusside ($Na_2[Fe(CN)_5NO]\cdot 2H_2O$). Sodium nitroprusside is toxic and can cause bodily irritation. Please read the chemical's MSDS before using.

4.4 The chemicals used to prepare the sodium phenolate reagent include crystalline phenol (C_6H_5OH) and sodium hydroxide (NaOH). Crystalline phenol could be fatal or toxic with exposure by breathing, contact, etc. Sodium hydroxide highly corrosive and can cause bodily irritation. Please read the chemical's MSDS before using.

4.5 Concentrated Sulfuric Acid is highly corrosive and can cause bodily irritation and burns. Please read the chemical's MSDS before using.

4.6 The chemicals used to prepare the sulfanilamide reagent include 85% phosphoric acid (H3PO4), sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride (NED). Phosphoric acid is corrosive and is an irritant. Sulfanilamide and NED can cause bodily irritation.

4.7 The chemicals used to prepare the ammonium chloride buffer reagent include ammonium chloride (NH_4Cl), disodium ethylenediamine tetraacetic acid dehydrate ($Na_2EDTA\ 2H_2O$), and 15 N sodium hydroxide solution. All chemicals should be treated as hazardous and handled with care.

5. Procedure:

5.1 **Pre-run checklist: (For any of the three assays)**

5.1.1 Prepare samples.

5.1.2 Check to see that the reagent waste bottles are not full. If so, properly dispose of the waste.

5.1.3 Check to see if there is enough reagent solution in each bottle to run the samples. Typically, half a reagent bottle will run approximately 200 samples. It is good practice to simply ensure that the bottle is at least half-full (reagents are kept in the refrigerator outside of the room the Lachat is in).

5.1.4 Change out the DI water in the 3000mL flask (at least half-full).

5.1.5 Make sure that the standards are no lower than 10mL. If so add more standard solution.

5.2 **Approach to analyze a set of samples for the determination of ammonia:**

5.2.1 Turn on the computer and enter the appropriate login name and password.

5.2.2 Turn on the power to all the machine components:

5.2.2.1 The Autosampler (red switch on the back)
5.2.2.2 The Pump (green switch on the back)
5.2.2.3 The System Unit (black switch on the back)

5.2.3 Open the Omnion 3.0 software.

5.2.4 In the menu toolbar, select Run→ Open and open the ammonia default method file. Make changes to the sample list by adding or deleting samples according to the number of samples to run. This is done with right-click options.

5.2.5 Prepare reagents if needed following the steps in section 4.

5.2.6 Place the tube lines (stored in a dry bag) coming from the ammonia manifold each in their own cartridge and snap it onto the pump. There are seven tube lines total and each are labeled with tags and are color coded. For convenience, from top to bottom the colors should be ordered:

5.2.6.1 Green-Green (the sample tube)
5.2.6.2 Green(with white)- Green(with white) (the carrier - DI water in 3000mL flask)
5.2.6.3 Orange(with white)- Orange(with white) (the reagent – sodium nitroprusside)
5.2.6.4 Orange(with white)- Orange(with white) (the reagent – sodium phenolate)
5.2.6.5 Red(with white)- Red(with white) (the reagent – sodium hypochlorite)
5.2.6.6 White(with black)-White(with black) (the buffer – disodium ethylenediamine tetraacetic acid(Na2EDTA) and sodium hydroxide (NaOH)
5.2.6.7 Purple-Purple (the rinse – DI water 3000mL flask)

5.2.7 Tighten the cartridges by moving the levers on left hand side of each cartridge entirely to the right.

5.2.8 Place all the tube lines in the 10% Hydrochloric Acid rinse (enabling the ends to be submersed in the rinse). This will get rid of any residual traces of ammonia that could still be in the lines from the last run.

5.2.9 Press the Manual Run/Stop button on the pump and rinse the lines for about five minutes to remove all air bubbles. During this time, check for leaks and ensure that there are no air bubbles in the lines. If leaks have occurred, turn off the pump, and address the problem or seek help.

5.2.10 When the water is freely flowing through the lines and all the air bubbles have ceased to flow through, turn the pump off by pressing the Manual Run/Stop button.

5.2.11 Once the pump is off, place all the tube lines in the 3000mL flask of rinse DI water (enabling the ends to be submersed in the water).

5.2.12 Press the Manual Run/Stop button on the pump and rinse the lines to remove the rest of the hydrochloric acid rinse.

5.2.13 Let it run for about two minutes, then turn the pump off by pressing the Manual Run/Stop button.

5.2.14 Once the pump is off, place the different tube lines into the proper solutions (buffer, reagent, rinse, etc.). Refer back to Step 5.2.6 of this procedure to assign where each tube line should go. Note: To prevent the reagent's exposure to the atmosphere, set the cap on the bottle opening avoiding pinching the tubes.

5.2.15 Turn the pump back on (Manual Run/Stop button) and let the reagents flow through the lines for about 2 minutes before proceeding to the next step.

5.2.16 Press the Preview button (eye icon) and check the background for approximately 2 minutes. This should be displayed in the Channel 1 – Detector 3 window. If the background is not uniform and fairly level either address the problem or seek help. The voltage on the y-axis of the preview graph should be much less than 0.6V (~0.2V). If this is not the case, address the problem or seek help.

5.2.17 Once background stability has been achieved, click the Stop button (red dot icon).

5.2.18 Load the sample tray containing samples to be analyzed onto the autosampler tray. Make sure the sample tray is securely in place (it should click in and not move around).

5.2.19 Open the standard vials (50mL centrifuge tubes with standards from step 4.2.1) and ensure they are in the correct locations.

5.2.20 When the sample tray is in place and the standards are ready, click the Start button (arrow icon).

5.2.21 Standby and watch to ensure the first peak hits its target height (~3.0V) and that it is analyzed correctly (the blue analyzed area should contain two perpendicular red lines; one at the base of the curve and the other going up to the peak of the curve).

5.2.22 Once the samples are complete, click Tools→Custom Report, then Report→Save as RTF and choose the appropriate name (use the date the samples were run; MMDDYYYY) and folder to save it under (should be the ammonia folder).

5.2.23 Follow the shutdown procedure.

5.3 **Approach to analyze a set of samples for the determination of orthophosphates:**

5.3.1 Turn on the computer and enter the appropriate login name and password.

5.3.2 Turn on the power to all the machine components:

5.3.2.1 The Autosampler (red switch on the back)

5.3.2.2 The Pump (green switch on the back)

5.3.2.3 The System Unit (black switch on the back)

5.3.3 Open the Omnion 3.0 software.

5.3.4 In the menu toolbar, select Run→ Open and open the ammonia default method file. Make changes to the sample list by adding or deleting samples according to the number of samples to run. This is done with right-click options.

5.3.5 Prepare reagents if needed following the steps in section 4.

5.3.6 Place the tube lines (stored in a dry bag) coming from the ammonia manifold each in their own cartridge and snap it onto the pump. There are seven tube lines total and each are labeled with tags and are color coded. For convenience, from top to bottom the colors should be ordered:

5.3.6.1 Green-Green (the sample tube)

5.3.6.2 Orange-White (the reagent – Ascorbic Acid)

5.3.6.3 Orange-Orange (the reagent – Molybdate color reagent)

5.3.6.4 Yellow- Yellow (the carrier– 3000-mL flask with DI water)

5.3.6.5 Purple-Purple (the rinse – DI water 3000mL flask)

5.3.7 Tighten the cartridges by moving the levers on left hand side of each cartridge entirely to the right.

5.3.8 Place all the tube lines in the Sodium Hydroxide rinse (enabling the ends to be submersed in the rinse). This will get rid of any residual traces of phosphate that could still be in the lines from the last run.

5.3.9 Press the Manual Run/Stop button on the pump and rinse the lines for about five minutes to remove all air bubbles. During this time, check for leaks and ensure that there are no air bubbles in the lines. If leaks have occurred, turn off the pump, and address the problem or seek help.

5.3.10 When the water is freely flowing through the lines and all the air bubbles have ceased to flow through, turn the pump off by pressing the Manual Run/Stop button.

5.3.11 Once the pump is off, place all the tube lines in the 3000mL flask of rinse DI water (enabling the ends to be submersed in the water).
5.3.12 Press the Manual Run/Stop button on the pump and rinse the lines to remove the rest of the sodium hydroxide rinse.
5.3.13 Let it run for about two minutes, then turn the pump off by pressing the Manual Run/Stop button.
5.3.14 Once the pump is off, place the different tube lines into the proper solutions (buffer, reagent, rinse, etc.). Refer back to Step 5.3.6 of this procedure to assign where each tube line should go. Note: To prevent the reagent's exposure to the atmosphere, set the cap on the bottle opening avoiding pinching the tubes.
5.3.15 Turn the pump back on (Manual Run/Stop button) and let the reagents flow through the lines for about 2 minutes before proceeding to the next step.
5.3.16 Press the Preview button (eye icon) and check the background for approximately 2 minutes. This should be displayed in the Channel 3 – Detector 3 window. If the background is not uniform and fairly level either address the problem or seek help. The voltage on the y-axis of the preview graph should be much less than 0.6V (~0.2V). If this is not the case, address the problem or seek help.
5.3.17 Once background stability has been achieved, click the Stop button (red dot icon).
5.3.18 Load the sample tray containing samples to be analyzed onto the autosampler tray. Make sure the sample tray is securely in place (it should click in and not move around).
5.3.19 Open the standard vials (50mL centrifuge tubes with standards from step 4.2.2) and ensure they are in the correct locations.
5.3.20 When the sample tray is in place and the standards are ready, click the Start button (arrow icon).
5.3.21 Standby and watch to ensure the first peak hits its target height (~2.5V) and that it is analyzed correctly (the blue analyzed area should contain two perpendicular red lines; one at the base of the curve and the other going up to the peak of the curve).
5.3.22 Once the samples are complete, click Tools→Custom Report, then Report→Save as RTF and choose the appropriate name (use the date the samples were run; MMDDYYYY) and folder to save it under (should be the phosphate folder).
5.3.23 Follow the shutdown procedure.

5.4 **Approach to analyze a set of samples for the determination of nitrate/nitrite:**

5.4.1 Turn on the computer and enter the appropriate login name and password.
5.4.2 Turn on the power to all the machine components:
5.4.2.1 The Autosampler (red switch on the back)
5.4.2.2 The Pump (green switch on the back)
5.4.2.3 The System Unit (black switch on the back)
5.4.3 Open the Omnion 3.0 software.
5.4.4 In the menu toolbar, select Run→ Open and open the ammonia default method file. Make changes to the sample list by adding or deleting samples according to the number of samples to run. This is done with right-click options.
5.4.5 Prepare reagents if needed following the steps in section 4.
5.4.6 Place the tube lines (stored in a dry bag) coming from the ammonia manifold each in their own cartridge and snap it onto the pump. There are seven tube lines total and each are labeled with tags and are color coded. For convenience, from top to bottom the colors should be ordered:
5.4.6.1 Green-Green (the sample tube)

5.4.6.2 White-White (the buffer - ammonia chloride)
5.4.6.3 White-White (the carrier - DI water in 3000mL plastic jug)
5.4.6.4 White-White (DI water – DI water in 3000mL plastic jug)
5.4.6.5 Orange-White (the reagent - sulfanilamide)
5.4.6.6 Purple-Purple (the rinse – DI water 3000mL flask)

5.4.7 Tighten the cartridges by moving the levers on left hand side of each cartridge entirely to the right.

5.4.8 Place all the tube lines in the 3000mL DI water rinse (enabling the ends to be submersed in the rinse).

5.4.9 Make sure the cadmium column is offline (the picture on the nob are two parallel lines. They should be parallel to the column [=]).

5.4.10 Press the Manual Run/Stop button on the pump and rinse the lines for about 5 minutes to remove all air bubbles. During this time, check for leaks and ensure that there are no air bubbles in the lines. If leaks have occurred, turn off the pump, and address the problem or seek help.

5.4.11 When the water is freely flowing through the lines and all the air bubbles have ceased to flow through, turn the pump off by pressing the Manual Run/Stop button.

5.4.12 Once the pump is off, place the different tube lines into the proper solutions (buffer, reagent, rinse, etc.). Refer back to Step 5.4.6 of this procedure to assign where each tube line should go. Note: To prevent the reagent's exposure to the atmosphere, set the cap on the bottle opening avoiding pinching the tubes.

5.4.13 Turn the pump back on (Manual Run/Stop button) and let the reagents flow through the lines for about 2 minutes before proceeding to the next step.

5.4.14 Turn the cadmium column inline (the picture on the nob are two parallel lines. They should be perpendicular to the column [||]).

5.4.15 Press the Preview button (eye icon) and check the background for approximately 2 minutes. This should be displayed in the Channel 2 – Detector 2 window. If the background is not uniform and fairly level either address the problem or seek help. The voltage on the y-axis of the preview graph should be much less than 1.0V (~0.6V). If this is not the case, address the problem or seek help.

5.4.16 Once background stability has been achieved, click the Stop button (red dot icon).

5.4.17 Load the sample tray containing samples to be analyzed onto the autosampler tray. Make sure the sample tray is securely in place (it should click in and not move around).

5.4.18 Open the standard vials (50mL centrifuge tubes with standards from step 4.2.3.1 or 4.2.3.2 depending on if you want to determine nitrate/nitrite) and ensure they are in the correct locations.

5.4.19 When the sample tray is in place and the standards are ready, click the Start button (arrow icon).

5.4.20 Standby and watch to ensure the first peak hits its target height (~5V) and that it is analyzed correctly (the blue analyzed area should contain two perpendicular red lines; one at the base of the curve and the other going up to the peak of the curve).

5.4.21 Once the samples are complete, click Tools→Custom Report, then Report→Save as RTF and choose the appropriate name (use the date the samples were run; MMDDYYYY) and folder to save it under (should be the nitrate folder).

5.4.22 Turn the cadmium column offline (the picture on the nob are two parallel lines. They should be parallel to the column [=]).

5.4.23 Follow the shutdown procedure.

5.5 **Shutdown Procedure: (For all three assays)**

5.5.1 Turn off the pump (Manual Run/Stop button).

5.5.2 Remove the reagent lines from each reagent, Place all the tube lines in the rinse (either 10% Hydrochloric acid rinse [ammonia] or Sodium Hydroxide rinse [phosphates]) enabling the ends to be submersed in the rinse. If nitrate/nitrite assay was run skip to step 5.5.4.

5.5.3 Let it run for about 5 minutes, then turn the pump off by pressing the Manual Run/Stop button.

5.5.4 Once the pump is off, place the lines into the DI rinse water (3000mL flask). Re-cap all the reagents.

5.5.5 Re-cap the standards.

5.5.6 Turn the pump back on and rinse the lines for 5 minutes.

5.5.7 Turn off the pump (Manual Run/Stop button).

5.5.8 After the rinse, suspend the end of the tube lines above the DI water and pump the lines dry.

5.5.9 Once dry, place the end of the tube lines in a dry bag for storage.

5.5.10 Turn off the pump and release the pump tube cartridges by loosening the levers (opposite of step 5.2.7, 5.3.7, and 5.4.7) and push the button levers on the side of the pump to release the cartridges. Remove the cartridges from the tube lines and set aside.

5.5.11 Close the Omnion 3.0 software.

5.5.12 Safely remove the hardware (as done with thumb drives).

5.5.13 Turn off the autosampler, pump, and system unit.

5.5.14 Clean up samples and workspace.

END

SOP - Backcrossing *Chlorella sorokiniana*

1. **Introduction and Scope**
   1.1 This document describes the procedure for backcrossing to homozygosity all transgenic strains developed for the PACE project to stabilize the genetic modification.

2. **Materials:**
   2.1 1L sterile HS media
   2.2 1L sterile HS media minus ammonia
   2.3 Blue LED at 480 nm

3. **Procedure:**

   3.1 **Confirm single copy gene insertion:** Using digital PCR or Southern blot. Screen your transgenics for those that have only one copy of gene of interest. Transgenics with multiple copies of the gene of interest are to be discarded.
   3.2 **Initial cultures:** Grow 100 mL of cells in 250 mL Erlenmeyer flask started from a single colony (this is critical, do not use multiple colonies). Grow at 37 C in HS media using a 12 hr light/dark cycle to synchronize cells. Grow cells to a density of approximately 5 x 10^7 cells/mL.
   3.3 **Induce Mating Competency**: Harvest cells at 2 hours before solar dawn in light/dark cycle in dim (green safe) light by centrifugation in 50 mL blue cap sterile tubes at 1500 g for 5 mins at RT. Resuspend cell pellet with 50 mLs of HS media minus ammonia. Repellet cells as above. Resuspend cells in 50 ML of HS media minus ammonia.
   3.4 **Dark (-N) Gamete Induction Treatment:** Place cells in sterile 250 Erlenmeyer flask and wrap well with foil to exclude all light and grow for 24 hours on shaker.
   3.5 **Blue Light (480 nm) Treatment to Induce Mating:** After 6 hours growth in darkness in (-) N media take cells into dark room and illuminate each flask with blue LED light (1-2 µmol/m^2/s) for 5 minutes in darkness. Rewrap flask in foil to exclude all light and continue to grow on shaker for a total of 24 hours.
   3.6 **Spread cells on antibiotic resistance plates to get single colonies:** After 24 hours in darkness spread serial dilutions of cells on HS plate with ammonia and antibiotic to get single colonies that are antibiotic resistant.
   3.7 **Driving to homozygosity:** Repeat steps 1-4 at least three more times.
   3.8 **Confirm homozygosity for gene of interest:** Use digital PCR to get gene of interest copy number (2). When there are 2 copies you are done.

SOP- Gravimetric Method for Determination of Dry Weight (DW) and Ash Free Dry Weight (AFDW)

1. Introduction and Scope

1.1 This is a gravimetric method for directly determining the total dry weight and ash free dry weight of biomass from a known volume of algal culture. Because moisture content can vary drastically and significantly influence results, the procedure involves filtering a known volume of culture onto a pre-weighed filter, drying overnight in an oven, and combusting in a muffle furnace. Accurate measurements of sample volume and representative sampling from the cultivation system are essential.

1.2 This method measures the total biomass in a cultivation sample and thus may include not only the algal species of interest but other algal species, bacteria, zooplankton, miscellaneous contaminating species, dead cells, cell fragments, and debris. Ash determination, while accounting for inorganic, nonvolatile contamination, will not allow for discrimination in algal vs non-algal biomass quantification.

1.3 The use of ammonium formate as wash agent is critical to reduce the amount of salt from the culture medium that remains within the sample/on the filter and thus can contribute to the DW determined and lead to artificially high ash % that is not reflective of the true inorganic, non-volatile material that can accumulate in a pond over time.

2. Reagents, Materials, and Apparatus Needed

2.1 **Chemicals and Reagents:**

2.1.1 0.5 M ammonium formate (VWR #AA14517-30; CAS #540-69-2) Prepare solution by adding 31.5 g of ammonium formate per liter of deionized or milli-Q water and stir until dissolved.

2.1.2 DRIERITE® absorbent (desiccant) (VWR #EM-DX2515-3; CAS #7778-18-9 (97%) and CAS #7646-79-9 (3%), regenerated. To regenerate DRIERITE® desiccant, granules should be single layered on a metal baking sheet. Heat oven to exactly 230°C (450°F). Desiccant granules should be baked for $1^1/_2$ to 2 hours. When process is completed, the product is ready for re-use.

2.2 **Materials:**

2.2.1 Filter paper, 410 (5.5 cm)
2.2.2 Glass Microfiber Filter paper, 696, 4.7 cm (VWR #28333-139)
2.2.3 Forceps, pointed tips, clean and dry
2.2.4 Pipette, 5-10 mL (VWR #89125-310; Eppendorf Research plus variable volume pipette)
2.2.5 Pipette tips, 5-10 mL (VWR #89087-532)
2.2.6 150 mL beaker (or similar)
2.2.7 Aluminum foil
2.2.8 Aluminum weighing pans, 57 mm (VWR #25433-010)

2.2.9 Ball point pen or heat resistant marker (up to 500°C)
2.2.10 Glass Petri dish

2.3 **Apparatus**

2.3.1 Drying oven set to 105°C (or alternative drying at 60°C)
2.3.2 Muffle furnace set to 500°C
2.3.3 Vacuum desiccator, 230 mm (VWR #24988-197; Bel-Art vacuum desiccator)
2.3.4 Desiccator plate, porcelain, 230 mm (VWR #89038-070)
2.3.5 Filtering apparatus (VWR #97003-752; Pall six position vacuum manifold: VWR #28143-546; 6-150 mL magnetic filter funnel assemblies, 47 mm)
2.3.6 Vortex (optional)
2.3.7 Analytical Balance (Readability 0.0001g minimum, 0.00001g preferred)

3. ES&H Considerations and Hazards

3.1 Standard laboratory personal protective equipment shall be worn; lab coat, gloves (nitrile or latex) and eye protection while working with chemicals.

3.2 DRIERITE is harmful if swallowed, causes respiratory tract, eye and skin irritation, and is a possible cancer hazard.

3.3 Ammonium formate may be irritating upon inhalation, ingestion and exposure to skin or eyes.

4. Procedure:

4.1 Enough sample must be drawn to allow for the required replication and the minimum amount of biomass required per sample (target is 10 mg, minimum is 5 mg). As an example, if the culture density (AFDW) is expected to be 0.1 g/L, and AFDW is to be measured in triplicate, than a minimum grab sample pull from the cultivation system of 300 mL is needed to allow for 10 mg of biomass filtered per replicate.

4.2 Number the tabs of the desired number of aluminum weighing pans using a ball point pen. Make sure to leave an impression as the ink will burn off the aluminum weighing pans while ashing the glass microfiber filters (step #21). Alternatively, you can use heat resistant marker if it is able to withstand temperatures up to 500°C.

4.3 Pre-ash the glass microfiber filters by laying them in a glass Petri dish and placing the Petri dish in a 500°C muffle furnace overnight. This will allow for an accurate tare weight of the filters pre-sample combustion.

4.4 Remove the Petri dish from the muffle furnace and move the pre-ash filters to a 150 mL beaker using forceps. Cover the beaker with aluminum foil.

4.5 Place beaker containing the filters in a vacuum sealed desiccators containing charged desiccant. If the filters are not used by the end of the day, return them to a 105°C drying oven overnight.

4.6 When ready to use filters, slowly release the pressure from the desiccator to avoid shifting items inside, then remove the beaker containing the filters. Be sure to close the desiccator and return the vacuum to prevent desiccant or other items from absorbing moisture. **NOTE:** Close the desiccator and return the vacuum to prevent desiccant or other items from absorbing moisture. Recharge the desiccant if it is exposed to air for any extended period of time, and as needed.

4.7 Using forceps, place a filter on the analytical balance and record the "Filter ID" and the "Filter (g)" in the production spreadsheet. You may choose to stack related filters and cover samples with a weighing pan marked with sample identification.

4.8 Repeat step 4.7 until the desired number of filters have been weighed. **NOTE**: Be sure to use dry, clean forceps to handle filters and return remaining filters to the desiccator.

4.9 Check that the filtering apparatus is prepared. Verify that the waste collection flasks are emptied. Make sure that all tubing is connected properly (tubes from each end of apparatus to stopper in the respective waste collection flask and the tubing from the side of each flask to one of the vacuum nozzles). Check that each individual filter holder's stopcock is turned off (parallel to bench top). Turn the vacuum on.

4.10 Clean the apparatus by rinsing magnetic filtration funnel cups and filtration surfaces thoroughly with DI water. You may choose to scrub crevices with a sponge or paper towel.

4.11 Using forceps place one filter on each of the filter funnel bases and cover with the magnetic funnel. Make sure to align the correct aluminum tin with the correct filtration setup to avoid confusing filters. All filters should return to their original tin, which indicates the sample number.

4.12 Determine the volume of sample to be filtered. Target amount of biomass is 10 mg of biomass (absolute minimum is 5 mg). OD_{750nm} vs. AFDW correlations can be utilized to estimate current concentration of the biomass in the sample. Record the volume of sample filtered. **NOTE:** For dilute cultures (0.05 – 0.1 g/L) this can mean filtering as much as 100-200 ml of culture medium per replicate.

4.13 Wet filter surface with ~20 mL DI water immediately before dispensing your sample. Turn stopcocks on to rinse excess DI water through the filter.

4.14 Mix desired sample well using the Vortexer or inverting sample tube multiple times. Make sure the sample is completely homogenized.

4.15 Use a pipette to dispense the appropriate volume into the cups holding each of the filters assigned to the sample. Turn the stopcocks for those filter holders on (perpendicular to lab bench). Record the volume dispensed for each sample in the production spreadsheet ("mL Filtered").

4.16 When all liquid has filtered through, turn the stopcock off.

4.17 To rinse, use a squirt bottle to dispense approximately 20 mL of 0.5 M ammonium formate to each cup. Allow to sit briefly (at least 20 seconds) then turn stopcock on again. After all of the solution has filtered through, turn the stopcock off and repeat process until you have rinsed three times. At the end of the third rinse step do NOT turn the stopcock off. Instead, remove filtration cup with the vacuum on and use the squirt bottle to outline the edges of the filter with 0.5 M ammonium formate solution. Repeat three times.

4.18 When all of the ammonium formate solution has filtered through, turn the stopcock off and use forceps to remove the filter and return it to its respective foil weighing pan.

4.19 Repeat Steps 4.11-4.18 for all samples being tested.

4.20 Place foil weighing pans containing filters with biomass into the 105°C drying oven overnight, or for at least 12 hours. **NOTE:** If you have a strain with which you are concerned about volatile organics being lost at the higher temperature, you can employ an alternative method of drying samples using a 60°C drying oven for 48 hours.

4.21 Once the appropriate amount of time has elapsed, remove foil weighing pans with filters from the drying oven to a desiccator containing charged desiccant and allow cooling to room temperature (approximately 30 minutes).

4.22 When cool, use the same analytical balance to weigh each filter with dry biomass and record the result in the production spreadsheet as "Filter + Biomass (g)". Again, be sure to use dry, clean forceps to handle filters.

4.23 To ash the samples, place the aluminum weighing pans with filters in a 500°C muffle furnace for four hours.

4.24 Move the aluminum weighing pans with filters to a glass desiccator (do not use a plastic desiccator as it will melt). Place under vacuum and allow cooling to room temperature (~30 minutes).

4.25 When the aluminum weighing pans and filters have cooled, remove them from the desiccator and, using an analytical balance, weigh each filter and record the results "Filter + Ash (g)" in the productions spreadsheet. Again, be sure to use dry, clean forceps to handle filters.

5. Calculations

5.1 The units of dry weight (DW) are in grams per liter (g/L). This is calculated by finding the weight of biomass on the paper and multiplying by (1000/mL volume filtered).

$$Dry\ Weight = \left(Weight_{Dry\ Filter+\ Biomass} - Weight_{Dry\ Filter}\right) \times (1000 \div mL\ filtered)$$

5.2 The units of ash free dry weight (AFDW) are in grams per liter (g/L). This calculation takes in to consideration the contribution of the ash in the culture media/on the filter (e.g. residual salt not removed in the ammonium formate rinse) and subtracts it to achieve an ash free dry weight:

$$Ash\ Free\ Dry\ Weight = \left(Weight_{Dry\ Filter+\ Biomass} - Weight_{Dry\ Filter+Ash}\right) \times (1000 \div mL\ filtered)$$

5.3 %Ash calculation takes the grams of ash and divides it into the grams of algae biomass:

$$\%Ash = \left(Weight_{Dry\ Filter+\ Ash} - \ Weight_{Dry\ Filter}\right) / \left(Weight_{Dry\ Filter+\ Biomass} - Weight_{Dry\ Filter}\right) * 100$$

END

SOP- Clean Room Sampling Procedures

1. Introduction and Scope

1.1 This procedure describes how to sample cultures that are in 2x2 panels located in the clean room in ISTB3 264A1, 264A, the washroom and the greenhouse. Sampling schedules vary according to purpose of culture. Please refer to PI for sampling schedule.

2. Reagents, Materials, and Apparatus Needed

2.1 50-mL conical/centrifuge tubes with screw-top lids
2.2 50-mL syringe
2.3 Syringe container/beaker
2.4 Bleach
2.5 DI water
2.6 UV sterilized water
2.7 70% ethanol
2.8 Hose with detachable on/off nozzle
2.9 20 L carboy
2.10 pH meter
2.11 Aluminum foil
2.12 Data log and pen

3. ES&H Considerations and Hazards

3.1 **Sodium Hypochlorite (Bleach) 5%:** is corrosive, an irritant, a sensitizer, and is toxic to ingest. It may have mutagenic effects. It may cause burns if it comes in contact with skin, and inhalation may cause irritation to respiratory tract. If it comes into contact with eyes, it may cause severe inflammation. Bleach solution may emit toxic fumes when overheated. It is not combustible alone, but may ignite if in contact with combustibles and some organic materials. See MSDS.

4. Procedure:

4.1 Always wear a lab coat and nitrile/latex gloves in the lab. You should wear safety glasses when handling chemicals or biomass. Work in a well-ventilated area.

4.2 Use a sterile 50-mL syringe for each batch sampled or disinfect a previously used 50-mL syringe for at least 10 minutes in at least a 10% bleach solution. (We like to use one 50mL syringe per strain, bleaching between uses.)

4.3 Label one 50-mL conical/centrifuge tube for each tank sampled. Make sure you label the tank number, your name, the batch number (or other identifier), and the date sampled. **Note:** amount of each sample varies depending on the experiment.

4.4 Remove and rinse 50-mL syringe(s) with deionized (DI) water for at least 30 seconds. Any residual bleach will inhibit algae growth. Rinse syringe container/beaker with DI water until the container no longer smells of bleach. Cover disinfected container containing syringe with aluminum foil.

4.5 Bring syringe, centrifuge tubes, pH meter, and data log/binder into the clean room. After entering the clean room and before coming into contact with any tanks, change gloves and spray with 70% ethanol and rub gloves together for about 30 seconds to sanitize. If gloves come in contact with lab coat, pen, surfaces, or any other possible sources of contamination, spray gloves again with ethanol.

4.6 Only work with one strain/batch at a time. Change syringes, gloves and re-spray with ethanol before beginning to work with a second strain or batch.

4.7 Assess the status of the tanks. All tanks should look healthy. If there are tanks that look unhealthy (unhealthy tanks may look: bleached-out, dark-colored, with a disproportionate amount of biofilm or sedimentation or foaming, etc.) you should always single those tanks out and wait to touch them until ALL of the healthy-looking tanks are taken care of. Even if these tanks turn out to not be contaminated, you do not want to risk contaminating healthy tanks!

4.8 Turn off the aeration on each tank (except possibly contaminated ones) and check to see if they are still at 15L. Use UV sterilized water to volume up the tanks to the 15L mark. You may use the disinfected syringe container to transport the UV sterilized water. In this case, spray the syringe with 70% ethanol and wrap in aluminum foil until ready to use. Ethanol the nozzle for the UV sterilized water and the syringe container before filling with UV sterilized water.

4.9 Carefully move aside foil covering top of lid and crack lid of tank open, taking care not to touch the inner part of the tank with gloves. There may be condensation-just try to disturb the water as little as possible.

4.10 Volume up the tanks in order (except possibly contaminated ones), without worrying about fixing the lids and foil, as you will be sampling these in just a moment, and turn on the air after each water level reaches 15L. Ethanol your hands and the container between each tank.

4.11 All tanks should have aeration on and be mixing before you start sampling. You should take this time to record the time and date of sampling on your data sheet. Ethanol your hands before sampling.

4.12 Insert syringe into the tank about 1-2 inches below the surface of the algae and pull at least 45 mL of algae into the syringe.

4.13 Transfer collected algae into a labeled centrifuge tube.

4.14 Immediately insert pH meter into tube with algae and record pH reading. If pH is outside of the range of 7.0-8.5 then it should be readjusted after sampling (see step 17 for guidance).

4.15 Screw the cap onto the centrifuge tube. Replace lid on tank and fix aluminum foil. Make sure the aluminum foil completely covers the lid to minimize evaporation and possible splashing.

4.16 Make sure all algae are expelled from the syringe and then ethanol the syringe and your hands between each tank, and repeat steps 4.10-4.16 for all tanks (except possibly contaminated ones).

4.17 Now you can repeat steps 8-17 for the contaminated tanks.

4.18 When finished, rinse pH meter with DI water.

4.19 It is important for pH to remain uniform between tanks for uniform growth. A high or low pH can indicate an unhealthy culture, or a culture that is not growing. If pH is not uniform and between 7.0-8.5:

a. Check that air tubing is firmly attached into air square. Listen for hissing to indicate a leak. If leak is found, make adjustment or seek assistance. Increase or decrease airflow by using the red lever attached to the light rack.

b. If there is uniform aeration in tanks, but pH is still too high or too low, then the carbon dioxide (CO_2) mixture needs to be adjusted. Only technicians should perform these adjustments. CO_2 settings are as follows:

i. Flow meter should near the bottom of the flow range. Changes should not be made visually, but by turning the knob in ¼ turn increments.

ii. If the flow of CO_2 is in this range and pH is below 7.0, then CO_2 flow should be decreased.

iii. If the pH is above 8.5, then CO_2 flow should be increased. Make small adjustments, and recheck pH 1 hour after adjusting CO_2 flow. Readjust as needed until pH is in the correct range.

iv. On CO_2 tank regulator, CO_2 should be flowing below 20 psi. CO_2 tank should be replaced when below 200 psi.

4.20 Turn off the water valve at the UV sterilizer, dispel stale water from the hose lines into a carboy and clean any spills before leaving clean room.

END

SOP- Indoor Seed Production in Columns and Panels

1. Introduction and Scope

1.1 This document describes indoor seed production of algae species in 800 mL glass columns and 15 L 2x2 flat panel photobioreactors (PBRs) to be used for ATP^3 Unified Field Studies (UFS) and Advanced Field Studies (AFS). Equipment assembly and maintenance, and inoculation procedures are contained herein. The medium used for indoor seed production of fresh water strains is a modified BG-11 media while saltwater strains use a modified f/2 media recipe (see Media Recipe Protocols).

2. Reagents, Materials, and Apparatus Needed

2.1 **Materials: 800 mL Column Assembly**

- 2.1.1 800mL glass columns (supplied by ASU glass shop)
- 2.1.2 Capillary tubes (supplied by ASU glass shop)
- 2.1.3 #10 rubber stoppers with two holes drilled out
- 2.1.4 Cotton balls
- 2.1.5 Forceps
- 2.1.6 Water in a spray bottle
- 2.1.7 250mL, 500 ml and 1000 ml graduated cylinders
- 2.1.8 800 mL glass column racks
- 2.1.9 70% ethanol solution in spray bottle
- 2.1.10 Aluminum foil
- 2.1.11 Laminar flow hood
- 2.1.12 0.2 micron in-line air filters (VWR part# 28145-477)
- 2.1.13 Versilic SPX-50 Silicone Tubing (ID 3/16” OD 5/16” Wall 1/16”)
- 2.1.14 Python Products Air Line Tubing, 500 ft.
- 2.1.15 Bunsen burner or torch

2.2 **Materials: 2’x2’ Panel Assembly**

- 2.2.1 Assembled 2x2 panel, PVC aeration square, and panel lid
- 2.2.2 Two, 6” lengths of disinfected ½” OD aeration tubing
- 2.2.3 0.2 micron in-line air filter

2.3

3. ES&H Considerations and Hazards

3.1 Standard laboratory personal protective equipment shall be worn; lab coat, gloves (nitrile or latex) and eye protection while working with chemicals. Special care should be given when handling sodium nitrate because it is a strong oxidizer. Contact with other materials may cause fire. Harmful if swallowed or inhaled. May cause irritation to skin, eyes and respiratory tract. Special care should be given when handling sodium hydroxide as it is a strong corrosive.

4. Procedures:

4.1 Preparation of 15 L Modified f/2 and BG-11 Media in Carboy for Columns

4.1.1 Place a magnetic stir bar in a 20 L Nalgene carboy.

4.1.2 Fill carboy with 13 liters of filtered (5 um) fresh water, seawater or artificial seawater (Measure 497 grams of artificial sea salt and add to carboy filled with fresh water). Water source is strain dependent.

4.1.3 Place the carboy on a stir plate and begin stirring.

4.1.4 Based on media and nitrogen source desired, add nutrients (nitrogen and phosphorous stock solution, trace metal stock solution, carbonate stock solution, etc.) to the carboy as per Table 1 below. See Modified f/2 Media Recipe and Modified BG-11 Media Recipe Protocol for stock solution recipes.

4.1.5 When everything has dissolved fully, remove the carboy from the stir plate, and remove the magnetic stir bar.

4.1.6 Bring to a final volume of 15 L using seawater or DI water.

4.1.7 Autoclave and store at room temperature.

Table 1: 15 L f/2 and BG-11 Media Recipes for Various N Sources

Media	N:P, ml	Trace, ml	Carbonate, ml	Iron, Mg, Ca; ml
f/2: Sodium Nitrate	133	15	0	
f/2: Ammonium Chloride	133	15	187.5	
BG-11	75 N stock, 3.75 P stock	3.75	3.75	3.75

4.2 Preparation of Sterile 800 mL Columns

4.2.1 Each column must have volumes of 200mL, 400mL, and 800mL clearly marked on the outside of the column to aid in inoculum preparation.

4.2.2 Starting with a clean column, thread a capillary tube through one of the drilled holes in a #10 rubber stopper. Wet the surfaces to facilitate this movement. **Be extremely careful as the capillary tubes are fragile and can easily break, presenting a risk for injury.**

4.2.3 Use forceps to plug the second hole in the stopper with one or two pieces of cotton.

4.2.4 Fill the column with ~5 ml of water.

4.2.5 Place the assembled rubber stopper and capillary tube into a clean 800mL column, making sure to adjust the length of the capillary tube so its end is just slightly off the bottom of the column when the rubber stopper is inserted in the column.

4.2.6 Attach a 5” piece of Versilic SPX-50 silicone tubing (3/16”) to the top of the capillary tube.

4.2.7 Remove the stopper so the 800mL column is no longer airtight, to prevent columns exploding in the autoclave.

4.2.8 Fold over the aquarium tubing, so that it sits between the rubber stopper and glass column.
4.2.9 Wrap the head of the column up in aluminum foil and seal with a piece of autoclave tape.
4.2.10 Repeat steps 4.4.2 thru 4.4.9 for each column to be inoculated.
4.2.11 Autoclave the columns for at 100 °C for 30 minutes and store at room temperature.

4.3 <u>**Procedure for 800mL Column Inoculation/Splits**</u>
***REMEMBER: Exercise proper aseptic technique when working with open cultures in the laminar flow hood. Any time a column is opened, the lip must be flame-sterilized before and after transferring algae inoculum.**

4.3.1 **Overview:** One 800mL glass column of algae inoculum will seed one 2x2 panel reactor. As seed production for ATP^3 will involve batches of 14 2x2s, inoculum will need to be progressively divided from one column to 16 columns (14 to inoculate 2x2s, and two to remain behind for future inoculations). One seed column is sufficient to inoculate 4 columns when the AFDW of 0.8 g/L or higher has been reached (note the AFDW can be an indirect estimate based OD750 using an OD750 versus AFDW correlation curve). The minimum target starting concentration after splitting is an AFDW of 0.2 g/L. Four columns can subsequently be divided into 16 columns when they reach an AFDW of at least 0.8 g/L. This procedure will provide 16x800mL columns - 14 to inoculate 2x2s, one as inoculum for future column divisions, and one as backup. Starting at 0.2 g/L or higher in the initial four columns should allow for 16 columns at sufficient density to inoculate panels in ~12-15 days from first split from a single column.
4.3.2 Always wear lab coat and latex or nitrile gloves while working in the lab. Safety goggles should be worn when working with biomass or chemicals.
4.3.3 Before touching anything in the clean room, put on new nitrile or latex gloves to avoid cross-contamination of algae. Clean hands with a spray bottle of 70% ethanol. Entire procedure should be followed for only one set of cultures at a time to avoid cross-contamination.
4.3.4 Turn off aeration to the 800mL column of algae inoculum.
4.3.5 Disconnect the top segment of aquarium tubing, making sure to leave the 0.2micron air filter attached to the column during transport to the laminar flow hood to protect the inoculum from airborne contaminants.
4.3.6 Place the inoculum on a rack on a cart and transport to laminar flow hood. Make sure the column rack has enough empty spaces to accommodate 16 columns.
4.3.7 Make sure the inoculum, sterilized 800mL columns, and column rack are all within reach while working in the hood.
4.3.8 Place the appropriate growth media carboy for the strain and treatment in the laminar flow hood.
4.3.9 Turn on the hood.

4.3.10 Spray all surfaces inside the hood with 70% ethanol and wipe clean with paper towel.
4.3.11 Turn on the gas line and light the Bunsen burner using a striker, or other lighter. Shield the flame from the airflow using a Bunsen burner shield to keep it from blowing out.
4.3.12 Spray gloves with 70% ethanol.
4.3.13 Make sure the column containing the inoculum is at 800mL. If it is short, top it off with autoclaved DI water under the laminar flow hood. Do this by opening the column, flame-sterilizing the lip, adding autoclaved DI water to 800mL, and finally flame-sterilizing the lip again before putting the rubber stopper and capillary tube back in place. Do this by using the capillary tube as a rest and rotating the upper lip of the column in the flame for 5-10 seconds. Make sure not to touch the stopper or capillary tube to any surface other than the sterile column.
4.3.14 Place the inoculum back on the rack. If any DI water was added, use an air nozzle with aquarium tubing attachment to mix 800 mL column by bubbling about 30 seconds through the filter on the top of the capillary tube apparatus.
4.3.15 In the hood, remove the aluminum foil cover of your first empty 800mL column.
4.3.16 Open and flame-sterilize the lip of the column as in step 4.3.13.
4.3.17 Place and hold the bottom of the empty column between your legs, resting the stopper ajar on the lip of the column while angling the column into the hood.
4.3.18 Open and flame-sterilize the lip of the column containing the inoculum as in step 4.3.13.
4.3.19 Carefully pour inoculum into the empty column to the 200mL marking.
4.3.20 Flame-sterilize the lip of the column containing the inoculum as in step 4.3.13., replace the stopper, and set it aside on the rack.
4.3.21 Flame-sterilize the lip of the column that contains 200mL of algae as in step 4.3.13., and add appropriate sterile media for the strain and treatment up to the 800mL fill line.
4.3.22 Flame-sterilize the lip of the column and replace the stopper as in step 4.3.13.
4.3.23 Attach a clean 0.2 micron in-line air filter to the column. Do this by connecting a short piece of Topfin airline tubing (small tubing) to the outlet of the filter to bridge the size gap between the connection on the filter and the Versilic aquarium tubing already connected to the column.
4.3.24 Return 800mL column to the rack.
4.3.25 Repeat steps 5.4.15-5.4.24 three more times, resulting in four identical columns of inoculum.
4.3.26 Turn off the gas and Bunsen burner and clean any spills with ethanol.
4.3.27 Make sure that you clean gloves again with ethanol before handling each column when returning the columns to the rack.
4.3.28 Place the column rack back on one shelf of the ATP^3 light racks, reconnect airline from each column to the manifold, and turn on air/CO_2.
4.3.29 If a column pH falls below 7.0, add 10 ml of the sodium carbonate solution (4.2.5) using sterile techniques.

4.4 **Detailed Procedure for Inoculation of 2'x2' Panel from an 800 mL Culture**

4.4.1 Transport a ready-to-use 2'x2' panel to clean room for inoculation.

4.4.2 Transfer the 2'x2' panel to the culture rack and line up the back edge of the tank approximately four inches from the light bulbs.

4.4.3 Fill 2'x2' panel with 14.2 liters of filtered (5 um) or UV sterilized water (but if using seawater, you may add directly since you will not be adding salt directly in step 4.4.6).

4.4.4 Lightly agitate the water with the PVC aeration square to begin dissolution. The salt will completely dissolve once the PVC aeration square is connected to the air/CO_2 line on the culture rack and mixing begins.

4.4.5 Check every panel for dechlorination with chlorine test strips.

4.4.6 Based on strain and treatment, add the appropriate nutrients or salt if creating artificial salt (add 497 grams of artificial sea salt to each 2x2 panel filled with fresh water) to each panel as per Table 1 above using sterile pipettes and graduated cylinders.

4.4.7 Place a clean lid on top of the 2'x2' panel.

4.4.8 Spray gloves with 70% ethanol and rub hands together for 15 seconds.

4.4.9 Assemble the in-line air filter by connecting the two pieces of aeration tubing to the air filter, and attach the assembly to the PVC square and air valve on the culture rack.

4.4.10 Adjust airflow to a moderate level, and ensure all tubing is securely fastened. Culture media should not be splashing out of tanks, but aeration should be strong enough so cells will not settle on the bottom of the tank. Allow 30 seconds for media to completely mix or 15 minutes for salt to dissolve before adding inoculum.

4.4.11 Remove 800-mL column containing clean inoculum from the column rack and carefully pour into 2'x2' panel, making sure the column does not touch the 2'x2' panel or panel lid.

4.4.12 Turn on the lights on the platforms holding the 2'x2' panel.

4.4.13 Place a piece of laboratory tape on the front of the panel with identification information (strain, inoculation date, panel number, initials).

4.4.14 After 30 seconds of mixing, take an initial sample following the AzCATI Clean Room Sampling Procedure.

4.4.15 Cover each panel tightly with foil to reduce evaporation and protect against airborne contaminants. Use two small pieces of foil (about 5" wide) to encircle the opening where the PVC air square meets the lid, and one larger piece (about 18") to span the length of the lid. Fold over any extra length of the foil around and under the panel's upper lip.

4.4.16 Clean any spills before leaving clean room.

*****ATTENTION*** Throughout this procedure take utmost care to keep gloves clean by spraying occasionally with 70% ethanol, and avoid contacting inner surface of the 2x2 panel and panel lid with any potential sources of contamination. Note that the tank**

material is susceptible to crazing when contacted by organic solvents such as ethanol. Avoid any prolonged contact on tank surfaces.

END

SOP- Modified BG-11 Media

1. Introduction and Scope

1.1 This document describes modified BG-11 nutrient production methods for indoor seed and outdoor pond production of fresh water algae species to be used for ATP^3 Unified Field Studies (UFS) and Advanced Field Studies (AFS). Individual stock solutions are to be replenished as needed.

2. Reagents, Materials, and Apparatus Needed

2.1 **Chemicals and Reagents for modified BG-11 Media:**

- 2.1.1 Sodium nitrate ($NaNO_3$)
- 2.1.2 Potassium phosphate dibasic (K_2HPO_4)
- 2.1.3 Magnesium sulfate heptahydrate ($MgSO_4*7H_2O$)
- 2.1.4 Calcium chloride dehydrate ($CaCl_2*2H_2O$)
- 2.1.5 Ferric ammonium citrate ($C_6H_8OFeH_3N$)
- 2.1.6 Citric acid ($C_6H_8O_7$)
- 2.1.7 Disodium ethylenediaminetetraacetic acid ($Na_2EDTA*2H_2O$)
- 2.1.8 Boric acid (H_3BO_3)
- 2.1.9 Manganese chloride tetrahydrate ($MnCl_2*4H_2O$)
- 2.1.10 Zinc sulfate heptahydrate ($ZnSO_4*7H_2O$)
- 2.1.11 Copper sulfate pentahydrate ($CuSO_4*5H_2O$)
- 2.1.12 Sodium molybdate dihydrate ($Na_2MoO_4*2H_2O$)
- 2.1.13 Cobalt nitrate hexahydrate ($Co(NO_3)_2*6H_2O$)
- 2.1.14 Sodium carbonate (Na_2CO_3)

2.2 **Materials for modified BG-11 Media:**

- 2.2.1 (7) 2 L glass bottle
- 2.2.2 20 L Nalgene carboy (LDPE) capable of being autoclaved
- 2.2.3 Magnetic stir bars

3. ES&H Considerations and Hazards

3.1 Standard laboratory personal protective equipment shall be worn; lab coat, gloves (nitrile or latex) and eye protection while working with chemicals. Special care should be given when handling sodium nitrate because it is a strong oxidizer. Contact with other materials may cause fire. Harmful if swallowed or inhaled. May cause irritation to skin, eyes and respiratory tract. Special care should be given when handling sodium hydroxide as it is a strong corrosive.

4. Procedure:

4.1 <u>**Generalized Procedure to Prepare Stock Solutions**</u>

- 4.1.1 Prepare all stock solutions in clean glass bottles.
- 4.1.2 Each stock solution should be prepared and stored separately.

4.1.3 Fill a glass bottle approximately half full with Milli-Q (MQ) water and add a magnetic stir bar.

4.1.4 Place the bottle on a magnetic stir plate and begin agitating the water. Adding chemicals to the bottle while stirring promotes rapid dissolution.

4.1.5 Weigh the appropriate amount of chemical(s) and carefully add to the bottle as its contents are stirring. Use a funnel to aid in transfer if necessary. If a funnel is used, rinse completely with MQ water from a squirt bottle.

4.1.6 Once the chemicals are completely dissolved, remove the bottle from the stir plate and remove the magnetic stir bar.

4.1.7 Bring to the final volume using MQ water.

4.1.8 Autoclave all stock solutions and label each with chemical(s) contained, CAS#, NFPA information, date, and initial. Store at room temperature.

4.2 <u>**Preparation of BG-11 Stock Solutions**</u>

4.2.1 Note that for all of the following it is best to start with a lower volume of MQ water than the final volume calls for and then finish by adding more to reach the final volume after adding chemicals. For all stock solutions, a stir bar can be added to the bottle which is then placed on a stir plate until all solids have dissolved.

4.2.2 **Indoor sodium nitrate stock solution (BG-11 1)**

4.2.2.1 Dissolve 600 grams sodium nitrate ($NaNO_3$) in MQ water to a total volume of 2 liters.

4.2.2.2 Autoclave and store at room temperature.

4.2.2.3 Use 5 ml/L culture water.

4.2.3 **Indoor potassium phosphate stock solution (BG-11 2)**

4.2.3.1 Dissolve 320 grams potassium phosphate dibasic (K_2HPO_4) in MQ water to a total volume of 2 liters.

4.2.3.2 Autoclave and store at room temperature.

4.2.3.3 Use 0.25 ml/L culture water.

4.2.4 **Outdoor combined N and P stock solution (N:P 20:1)**

4.2.4.1 Dissolve 6.375 kg of $NaNO_3$ and 650 grams K_2HPO_4 in MQ water to a total volume of 15 liter. The carboy does not need to be sterilized, but should be stored in air conditioned, low light conditions.

4.2.4.2 Use 1 ml stock/L culture water for initial inoculation. Reference pond protocols for feed levels post harvests.

4.2.5 **Indoor and outdoor magnesium sulfate stock solution (BG-11 3)**

4.2.5.1 Dissolve 600 grams magnesium sulfate heptahydrate ($MgSO_4*7H_2O$) in MQ water to a total volume of 2 liters.

- 4.2.5.2 Indoor stock solutions: autoclave and store at room temperature. Outdoor stock solutions: do not need autoclaved, store in air conditioned, low light condition.
- 4.2.5.3 Use 0.25 ml/L culture water.

4.2.6 **Indoor and outdoor calcium chloride stock solution (BG-11 4)**

- 4.2.6.1 Dissolve 288 grams calcium chloride dihydrate ($CaCl_2*2H_2O$) in MQ water to a total volume of 2 liters.
- 4.2.6.2 Indoor stock solutions: autoclave and store at room temperature. Outdoor stock solutions: do not need autoclaved, store in air conditioned, low light condition.
- 4.2.6.3 Use 0.25 ml/L culture water.

4.2.7 **Indoor and outdoor citric acid and ferric ammonium citrate stock solution (BG-11 5)**

- 4.2.7.1 Dissolve 48 grams of citric acid ($C_6H_8O_7$) and 48 grams ferric ammonium citrate ($C_6H_8OFeH_3N$) in MQ water to a total volume of 2 liters.
- 4.2.7.2 Indoor stock solutions: autoclave and store in a refrigerator or cold room at 40° F. Outdoor stock solutions: do not need to be autoclaved, store in a refrigerator or cold room at 40° F.
- 4.2.7.3 Use 0.25 ml/L culture water.

4.2.8 **Indoor and outdoor trace metal stock solution (BG-11 6)**

- 4.2.8.1 Fill a 2 L glass bottle approximately 75% full with MQ water.
- 4.2.8.2 Add a stir bar and place on a stir plate.
- 4.2.8.3 Add 8.0 grams disodium ethylenediaminetetraacetic acid ($Na_2EDTA*2 H_2O$). Make sure to add the EDTA first as it will act as a chelator for the other metals.
- 4.2.8.4 Add 22.88 grams boric acid (H_3BO_3).
- 4.2.8.5 Add 14.48 grams manganese chloride tetrahydrate ($MnCl_2*4H_2O$).
- 4.2.8.6 Add 1.76 grams zinc sulfate heptahydrate ($ZnSO_4*7H_2O$).
- 4.2.8.7 Add 3.12 grams sodium molybdate dihydrate ($Na_2MoO_4*2H_2O$).
- 4.2.8.8 Add 0.632 grams copper sulfate pentahydrate ($CuSO_4*5H_2O$).
- 4.2.8.9 Add 0.3952 grams cobalt nitrate hexahydrate ($Co(NO_3)_2* 6H_2O$).
- 4.2.8.10 Once everything has dissolved, remove the stir bar and bring to a total volume of 2 liters using MQ water.
- 4.2.8.11 Autoclave and store at room temperature.
- 4.2.8.12 Scale this recipe to 20 L to produce a carboy for outdoor production use. The carboy does not need to be sterilized, but should be stored in air conditioned, low light conditions.
- 4.2.8.13 Use 0.25 ml/L culture water.

4.2.9 **Indoor and outdoor sodium carbonate stock solution (BG-11 7)**

4.2.9.1 Dissolve 160 grams sodium carbonate (Na_2CO_3) in MQ water to a total volume of 2 liters.
4.2.9.2 Autoclave and store at room temperature.
4.2.9.3 Use 0.25 ml/L culture water.

END

SOP- Modified f/2 Media

1. Introduction and Scope

1.1 This document describes modified f/2 nutrient production methods for indoor seed and outdoor pond production of marine algae species to be used for ATP^3 Unified Field Studies (UFS) and Advanced Field Studies (AFS). Individual stock solutions are to be replenished as needed.

2. Reagents, Materials, and Apparatus Needed

2.1 **Chemicals and Reagents for modified f/2 Media:**

- 2.1.1 Sodium nitrate ($NaNO_3$) or Ammonium Chloride (NH_4Cl)
- 2.1.2 Sodium phosphate monobasic (NaH_2PO_4)
- 2.1.3 Sodium carbonate (Na_2CO_3)
- 2.1.4 Sodium hydroxide (NaOH, can source 1 M solution if preferred)
- 2.1.5 Disodium ethylenediaminetetraacetic acid ($Na_2EDTA*2\ H_2O$)
- 2.1.6 Iron (III) chloride hexahydrate ($FeCl_3*6H_2O$)
- 2.1.7 Copper (II) sulfate pentahydrate ($CuSO_4*5H_2O$)
- 2.1.8 Sodium molybdate dihydrate ($Na_2MoO_4*2H_2O$)
- 2.1.9 Zinc (II) sulfate heptahydrate ($ZnSO_4*7H_2O$)
- 2.1.10 Cobalt chloride hexahydrate ($CoCl_2*6H_2O$)
- 2.1.11 Manganese chloride tetrahydrate ($MnCl_2*4H_2O$)

2.2 **Materials for modified f/2 Media:**

- 2.2.1 (5) 1 L glass bottles
- 2.2.2 (2) 2 L bottles
- 2.2.3 (3) 20 L Nalgene carboys (LDPE) capable of being autoclaved
- 2.2.4 Magnetic stir bars

3. ES&H Considerations and Hazards

3.1 Standard laboratory personal protective equipment shall be worn; lab coat, gloves (nitrile or latex) and eye protection while working with chemicals. Special care should be given when handling sodium nitrate because it is a strong oxidizer. Contact with other materials may cause fire. Harmful if swallowed or inhaled. May cause irritation to skin, eyes and respiratory tract. Special care should be given when handling sodium hydroxide as it is a strong corrosive.

4. Procedure:

4.1 <u>**Generalized Procedure to Prepare Stock Solutions**</u>

- 4.1.1 Prepare all stock solutions in glass bottles.
- 4.1.2 Each stock solution should be prepared and stored separately.
- 4.1.3 Fill one 1L glass bottle approximately halfway with Milli-Q (MQ) or DI water and add a magnetic stir bar.

4.1.4 Place the bottle on a magnetic stir plate and begin stirring the water. Adding chemicals to the bottle while stirring ensures rapid dissolution.

4.1.5 Weigh appropriate amount of chemical(s) and carefully add to the bottle as it is stirring. Use a funnel to aid in transfer if necessary. If a funnel is used, rinse completely with MQ water from a squirt bottle.

4.1.6 Once the chemicals are fully dissolved, remove the bottle from the stir plate and remove the magnetic stir bar.

4.1.7 Volume up to the 1L mark with MQ water.

4.1.8 Autoclave all stock solutions and label each with chemical, name, CAS#, NFPA information and date. Store at room temperature.

4.2 <u>**Preparation of Stock Solutions**</u>

4.2.1 Note that for all of the following it is best to start with a lower volume of MQ water than the final volume calls for and then finish by adding more to reach the final volume after adding chemicals. For all stock solutions, a stir bar can be added to the bottle which is then placed on a stir plate until all solids have dissolved.

4.2.2 **Indoor combined nitrogen and phosphorus stock solution (N:P = 16:1)**

4.2.2.1 Stock solutions can be produced with either: sodium nitrate or ammonium chloride (see Table 1). Produce a separate stock solution for each nitrogen source.

4.2.2.2 Dissolve nitrogen source (see Table 1 for amounts) and 7.5 grams NaH_2PO_4 in MQ water to a total volume of 1 liter.

4.2.2.3 Autoclave and store at room temperature.

4.2.2.4 Use 8.87 ml stock/L culture water.

Table 1: 1 L Stock Solution Indoor Recipes for Various N Sources

N Source	Amount, g	Amount NaH_2PO_4, g
Sodium Nitrate Sln.	85	7.5
Ammonium Chloride Sln.	53.5	7.5

4.2.3 **Outdoor combined N and P stock solution (N:P 16:1)**

4.2.3.1 Stock solutions can be produced with either: sodium nitrate or ammonium chloride (see Table 2). Produce a separate stock solution for each nitrogen source.

4.2.3.2 Dissolve nitrogen source (see Table 2 for amounts) and 7.5 grams NaH_2PO_4 in MQ water to a total volume of 15 liter. The carboy does not need to be sterilized, but should be stored in an air conditioned, low light conditions.

4.2.3.3 Use 1 ml stock/L culture water for initial inoculation. Reference pond protocols for feed levels post harvests.

Table 2: 15 L Stock Solution Outdoor Recipes for Various N Sources

N Source	Amount, kg	Amount NaH_2PO_4, kg
Sodium Nitrate Sln.	6.375	0.5625
Ammonium Chloride Sln.	4.012	0.5625

4.2.4 **Primary trace metal stock solutions**

4.2.4.1 Each of the five primary trace metal stock solutions listed below should be prepared and stored in separate glass bottles.

4.2.4.2 Dissolve 9.8 grams $CuSO_4*5H_2O$ in MQ water to a total volume of 1 liter. Autoclave and store at room temperature.

4.2.4.3 Dissolve 6.3 grams $Na_2MoO_4*2H_2O$ in MQ water to a total volume of 1 liter. Autoclave and store at room temperature.

4.2.4.4 Dissolve 22.0 grams $ZnSO_4*7H_2O$ in MQ water to a total volume of 1 liter. Autoclave and store at room temperature.

4.2.4.5 Dissolve 10.0 grams $CoCl_2*6H_2O$ in MQ water to a total volume of 1 liter. Autoclave and store at room temperature.

4.2.4.6 Dissolve 180.0 grams $MnCl_2*4H_2O$ in MQ water to a total volume of 1 liter. Autoclave and store at room temperature.

4.2.5 **Trace metal stock solution**

4.2.5.1 Begin by dissolving 4.36 grams $Na_2EDTA*2H_2O$ in MQ water.

4.2.5.2 Then, add 3.15 grams $FeCl_3*6H_2O$ and 1.0 mL from each of the five primary trace metal stock solutions [$CuSO_4*5H_2O$ (4.2.3.2), $Na_2MoO_4*2H_2O$ (4.2.3.3), $ZnSO_4*7H_2O$ (4.2.3.4), $CoCl_2*6H_2O$ (4.2.3.5), $MnCl_2*4H_2O$(4.2.3.6)].

4.2.5.3 Continue to stir until fully dissolved.

4.2.5.4 Bring to a final volume of 1 liter with MQ water.

4.2.5.5 Autoclave and store at room temperature.

4.2.5.6 Scale this recipe to 20 L to produce a carboy for outdoor production use. The carboy does not need to be sterilized, but should be stored in air conditioned, low light conditions.

4.2.5.7 Use at 1 ml stock/L culture water.

4.2.6 **Indoor sodium carbonate stock solution (ammonium chloride nitrogen source only)**

4.2.6.1 Dissolve 80 g of sodium carbonate in 1 L DI or milli-Q water. Autoclave and store in an air conditioned space.

4.2.6.2 Use at 12.5 ml/L culture water. Use as needed to maintain pH above 7.0 in culture systems.

4.2.7 **Outdoor sodium hydroxide solution (ammonium chloride nitrogen source only)**

4.2.7.1 Sodium hydroxide is caustic. Wear appropriate PPE.

4.2.7.2 Dissolve 750 g of NaOH in 20 liters of DI water (this is a 1 M solution and can be purchased as a solution as an alternative to producing the solution if preferred). The carboy does not need to be sterilized, but should be stored in air conditioned, low light conditions.

4.2.7.3 Use at 0.25 ml/L culture water at inoculation. Use as needed to maintain pH above 7.0 in culture systems.

END

SOP-Optical Density

1. Introduction and Scope

1.1 This procedure describes how to prepare samples for optical density (OD) measurements and reading the absorbance at 750 nm on a spectrophotometer. OD readings must be between 0.1 and 0.6 to be accurate.

2. Reagents, Materials, and Apparatus Needed

2.1 Cuvette appropriate for your spectrometer (site-specific)
2.2 Cuvette caps or materials for an alternative mixing method (site-specific)
2.3 Spectrophotometer capable of reading 750nm wavelength (site-specific)
2.4 Pipettes capable of accurately dispensing 25 µL- 5 mL volumes (site-specific)
2.5 Pipette tips (site-specific)
2.6 Fresh f/2 culture media or seawater containing 35 g/L of sea salt
2.7 At least 2-5 mL of algae for each sample to be run (site-specific)

3. ES&H Considerations and Hazards

3.1 Wear proper personal protective equipment when handling samples.

4. Procedure:

4.1 Decide the dilution for your sample (see 'Sample Dilution Procedure' below). Samples with absorbance higher than 0.8 should be diluted. If the absorbance cannot be determined as, being higher than 0.8, then the samples should be run without dilution. A dilution may be performed later. Samples should be run in triplicate, with a separate dilution performed for each replicate if applicable.

4.2 Set up your spectrophotometer to run samples following the Instrument Operating Procedure for your instrument so you may run your samples immediately after they are prepared.

4.3 Mix algae sample thoroughly by capping and inverting the container several times before immediately pipetting appropriate volume (site-specific) into one cuvette. Or perform a dilution (see: below). If there are particles or other debris from an outdoor culture that may cause interference in your absorbance, try to avoid any debris.

4.4 Repeat step 4.3 for each of three replicates per sample (i.e. each sample must be prepared three separate times in three identical cuvettes).

4.5 Make sure you pipette a separate blank with your diluent.

4.6 When all samples have been dispensed into cuvettes, immediately analyze them on a spectrometer at 750nm following the procedure for your instrument. Be sure to mix vigorously before taking each absorbance reading. Use the cuvette containing only f/2 or seawater as a blank for absorbance to zero out before reading your samples. If any cuvettes sit for more than 10 minutes before analysis, you should prepare them again for accurate readings.

4.7 Plot absorbance over time on a graph to determine the qualitative density of your samples.

Sample Dilution Procedure (if necessary):

A few considerations before diluting a set of samples.

- The diluents should be the same fluid that your samples are in, and the same fluid as your blank. For example, f/2 media is used to dilute algae grown in f/2 media. Make sure, if you are using a re-used stock of the media that it does not have anything growing in it, as can happen if left on the benchtop under non-sterile conditions. Do not autoclave, but discard and make new media for your blank.

- Decide which dilution is needed to reach an absorbance between 0.2-0.8. For example, dense algae often require a 1:10 dilution.

- You may dispense and mix the diluents and algae samples in the cuvette (site specific) if you have a cap. Otherwise, pre-mix your diluents and algae samples separately in tubes capable of holding 1mL before pipetting the full amount into your cuvette.

A. Thoroughly agitate your samples and pipette the appropriate amount into duplicate test tubes or directly into your cuvette. (i.e., pipette 500 µL algae into 5 mL of f/2 or salt water for a 1:10 dilution)
B. Next pipette the appropriate amount of diluents into each test tube or cuvette. (i.e., pipette 5 mL of f/2 media or salt water for a 1:10 dilution). You should make three separate dilutions to run the sample in triplicate.
C. Record the µL algae and µL diluent dispensed on your spreadsheet.
D. Cap each tube or cuvette and invert several times to mix thoroughly. If you did not use the cuvette that you will read your sample in while making the dilution, dispense the appropriate amount of mixed, diluted sample into the appropriate vesicle.
E. Follow the procedure above from step 4.4 to finish analyzing your samples.

END